Table of Contents

How to use this guide

Once you have a finished phage genome sequence, you are ready to make predictions as to the locations and functions of the tRNA-coding and protein-coding genes. This guide will provide step-by-step instructions as to how to do this.

There are several different ways you can use this guide.

- Begin at **Section 1**, and proceed section by section through the entire guide. This approach will give you a complete understanding of the entire process of annotation and how each of the programs involved works. It's a lot of information, but hopefully you'll emerge from the other side far more knowledgeable about genes and gene calling.

- If you've already used the **DNA Master Quick Start Guide** to create an automated annotation, you can jump in at **Section 5**, and proceed from there. You'll be skipping some basics, but you can always refer back to relevant sections if needed.

- If you're eager to get straight to gene calling, you can perform an automated annotation using the **DNA Master Quick Start Guide** or **Section 4** of this guide, then proceed to **Section 8** which covers how to refine your automated annotation. References back to previous sections are provided so that you'll be able to locate all the information you need.

- If you're already an experienced annotator, and all you want to know is how to push the correct buttons to modify gene calls in DNA Master, **Section 9** is for you. It's an à-la-carte section of "How-To" functions.

- Finally, even if you're accustomed to using a different program to annotate phage genomes, you can use the Guiding Principles described in **Section 7.2** to see how we think about making the best possible gene calls in phage genomes.

A NOTE ON CLASSROOM PRAGMATICS

If you have a group of students annotating a single genome there are several different ways of organizing this activity. Assuming you have a class of around 20 students, there are two main considerations.

1. It works well for students to work in pairs, if possible using two computer stations. One of these can be set up to run DNA Master, while the other is set up to run Phamerator, as well as having other files (such as a six-frame translation) open.
2. You can organize students or groups of students such that:
    - All students annotate all of the genome. Upon completion, student groups (e.g. 5 groups of 4 students each) can each lead a discussion on a segment of the genome (i.e. 20%of it) aimed at resolving any differences found by different groups. The data are then compiled into a single DNA Master file.
    - Groups of students (e.g. 5 groups of 4 students) annotate a different segment of the genome (e.g. ~20%), followed by merging of the five DNA Master files into a single composite file. Instructions are provided in Stage 9 for doing this.

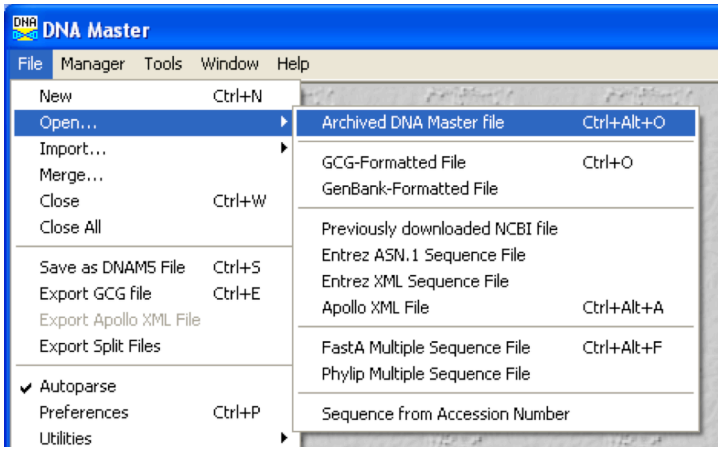
There are of course many other configurations and operational means of accomplishing your annotation. But it is helpful to keep in mind that the goal should be that all participants understand the full genomic context of the phage genome once the annotation is completed.

AN IMPORTANT NOTE ABOUT THIS GUIDE'S SYNTAX

In this guide, we will refer to menus and submenus as follows. If the command is:

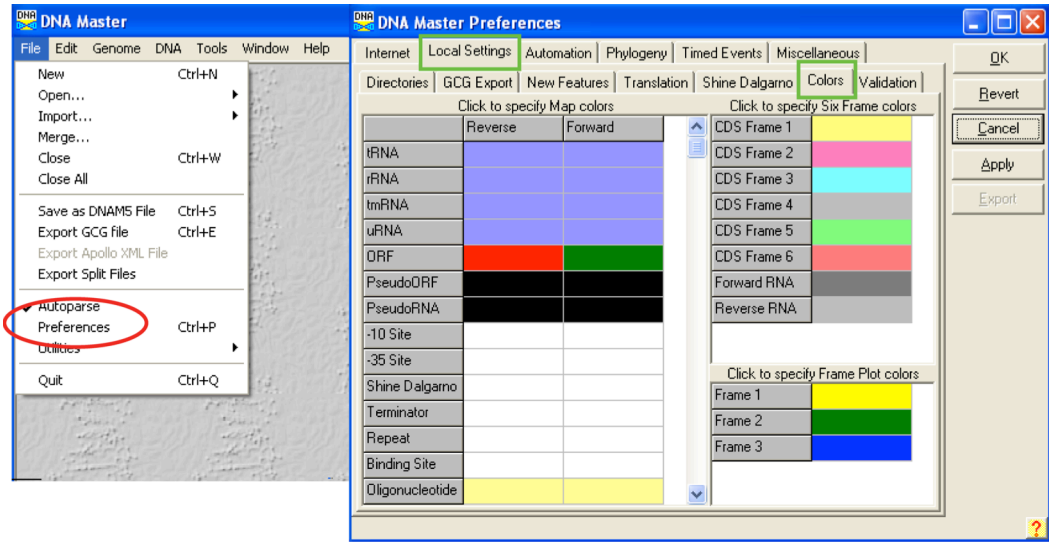
File → Open → Archived DNA Master file

this means that you should click on the **File** menu at the top, scroll down to the sub-menu (**Open**), and select the sub-sub-menu (**Archived DNA Master file**) that appears.



Tabs will be indicated by brackets, and sub-tabs will be shown by double brackets.

File → Preferences [Local Settings] [[Colors]]



1 Introduction to DNA Master

1.1 DNA Master overview

The key program you will use in your genome annotations is **DNA Master**. DNA Master is a DNA sequence editor and analysis package that combines, analyzes, and displays data from a variety of DNA analysis programs, including GeneMark, Glimmer, Aragorn, BLAST, and HHPred. It organizes and collates all of these data into various tables and forms and saves it a single file with a **.dnam5** extension.

1.2 Installation

This guide assumes that you have installed DNA Master and can open the program successfully. If this is not the case, please install DNA Master before continuing with this guide. System requirements and installation instructions are at http://phagesdb.org/DNAMaster/. DNA Master will need to be updated IMMEDIATELY!!! to complete the final step of the installation. To update, Open the program, go to Help, then click on Update DNA Master. The program requires a restart to accept all changes.

1.3 Quick Start Guide

The **DNA Master Quick Start Guide** is a useful tool when you are using DNA Master for the very first time and just want a quick look at basic functions. However, all parts of the Quick Start Guide are covered in more detail in this guide, so you may choose to use the Quick Start Guide as a future reference or a teaching tool.

1.4 DNA Master program structure

The various files, tables, and databases that DNA Master uses are a little complex, but a general understanding of the structure is important and will help prevent lost work.

The Feature Table

There are two important places DNA Master stores information about a genome annotation. The first, called the **Feature Table**, contains information about each feature (usually a gene) in a genome, including name, position, length, protein sequence, BLAST results, function, notes, etc. The Feature Table is a representation of the data stored for each feature. It is not a simple flat file, but rather a dynamic compilation of data. Within DNA Master, the data in the Feature Table for a particular genome can be viewed by going to the "**Features**" tab. When you **Post*** changes to your annotation, like changing a start position or adding a gene, you're altering the Feature Table.

* See **Section 9.3.1** for more on the importance of **Posting** changes.

The Documentation

The second place DNA Master stores information is the **Documentation**, accessible via the Documentation tab. This text contains much of the same information as is present in the Feature Table, but in a less human-friendly and more computer-readable format. Note that not all of the information from the Feature Table is contained in the Documentation Tab (e.g., amino acid sequence and BLAST hits are not present).

Interaction between the two

The Feature Table interacts with the Documentation as shown in **Figure 1.1**.

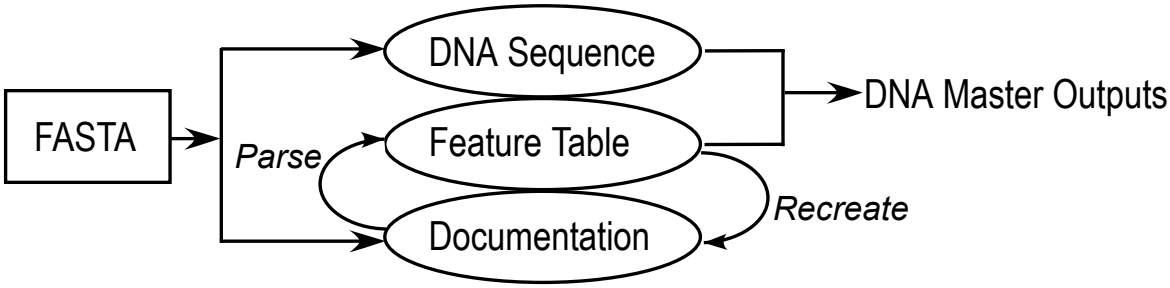


Figure 1.1

There are two functions—accessible through the Documentation tab—that control the interaction between the Feature Table and the Documentation:

Parse takes the contents of the Documentation and uses them to **OVERWRITE** the **Feature Table**. Parsing is done automatically by DNA Master when a genome is auto-annotated, but thereafter should be used rarely if ever. The danger is that you'll have posted data to the Feature Table that are not included in the documentation, and then when you Parse, those data will be lost.

Recreate takes the contents of the Feature Table, and uses them to **OVERWRITE** the **Documentation**. This will update the Documentation with changes you've posted, and thus serves as a helpful backup of some of your data. You will want to do this often.

IMPORTANT TO REMEMBER:

Using **Parse** may overwrite user-inputted data, and thus Parsing may be **harmful**.

Using **Recreate** will store some user-inputted data in a new location, and thus it's **helpful**.

As a safety feature, **Recreate** the **Documentation** often. If your .dnam5 file gets corrupted, you can use Documentation to build a new file. Frequent recreation of the Documentation seems to improve the stability of the overall file, and so we recommend recreation prior to saving the file each time.

Analysis programs running within DNA Master

As noted above, DNA Master runs a collection of programs that can assist in annotation and analysis of your phage genome. The following is a brief explanation of some of the key programs that DNA Master will be running for you, and some of their stand-alone versions that you will be using.

1.4.1 Glimmer

Glimmer (version 3.02) is a program that predicts the coding potential of open reading frames

(ORFs) based on a profile of frequently used codons found in the longest ORFs in the genome. DNA Master is set by default to use Glimmer in a heuristic way, meaning that it searches for potential coding regions (such as in long open reading frames) and then applies the nucleotide codon biases in those ORFs to search for other potential ORFs with similar biases. As such, it is not dependent on the use of externally defined parameters to determine coding potential. Glimmer also recognizes the use of TTG in addition to ATG and GTG as translation initiation (i.e. start) codons. It has very good predictive power for genes.

You will typically use Glimmer as a program that will run when you request DNA Master to perform an auto-annotation of your phage genome sequence and you will not be required to run it directly.

If you'd like to run Glimmer directly, it is available as a stand-alone program and is web-accessible at:

http://www.ncbi.nlm.nih.gov/genomes/MICROBES/glimmer_3.cgi

1.4.2 GeneMark

GeneMark also predicts genes within a genome sequence based on codon usage and codon potential. There are multiple forms of GeneMark tools developed by Mark Borodovsky and his colleagues that are useful to predict the genes in phage genomes. We will use at least two variations of this program. The ways to access this suite of programs has been altered in 2014 due to hacker misbehaviors. You will find that some of the previous versions are no longer available.

Heuristic Version included in DNA Master: GeneMark.hmm (version 2.0) provides a similar functionality to Glimmer. Its algorithms are different, however, and the joint use of Glimmer and GeneMark is a powerful combination for gene prediction. As with Glimmer, DNA Master runs GeneMark automatically within the Auto-Annotation function. Within DNA Master, the GeneMark that is run uses the heuristic model, in that it learns from the given genome what the codon usage preferences are in the longest ORFs and then applies this model to predict the remainder of the genes. GeneMark also takes into account potential ribosome binding sites when predicting gene start positions. This version of GeneMark will recognize TTG starts, importing them into DNAMaster.

Heuristic Version to obtain a graph of coding potential: GeneMarkS (Self training version for genomes >50 kb) is available on line at http://exon.gatech.edu/GeneMark/genemarks.cgi.

This version is similar to the one incorporated in DNA Master, however, you can obtain a graph of the coding potential by running the program through the website. This way, you can "see" what the numbers of the auto-annotation look like.

GeneMark with pre-trained model parameters: GeneMark.hmm (Version 3.25) is available online at http://exon.gatech.edu/GeneMark/gmhmmp.cgi .

In this internet browser-accessible version, the gene predictions are made using a codon usage model built from a previously annotated organism. GeneMark has many bacterial models available, and so for bacteriophage we pick a model based on the host organism. For the mycobacteriophage isolated on *M. smegmatis* mc^{2}155, we can use *Mycobacterium smegmatis* mc^{2}155 or one of the *Mycobacterium tuberculosis* strains listed.

This web version contains two key features that are useful for phage genome annotation:

- It allows you to specify the codon usage model from a bacterial host to use for gene

prediction, rather than generating a new model heuristically. A codon usage model for *Mycobacterium smegmatis* is available and can be selected to generate gene predictions in the phage genome based on the host's codon preferences. This sometimes allows you to find smaller genes that are not called during heuristic scans, but are likely to be reliable gene calls because they share codon preferences with the host. We refer to this output as the "**GeneMark-Smeg**" or "**GeneMark-TB**" output.

- It provides a graph (as a .pdf) of the gene predictions and coding potential. This is especially useful when you are determining gene starts.

A graph of the heuristic model can also be generated for comparisons. Use this version of the website http://opal.biology.gatech.edu/GeneMark/heuristic_hmm2.cgi

Note:

- Refer to Sean R. Eddy's paper "What is a hidden Markov model?" to contemplate the math behind GeneMark and Glimmer . Nature Biotechnology 22, 1315-16 (2004).
- Both Glimmer and GeneMark heuristic versions only use a **random sample** of the ORFs to generate results, so outputs are not strictly reproducible.
- The GeneMark.hmm version in DNA Master will include TTG starts, the graph from the prokaryote model (Version 3.25) does not.

1.4.3 Aragorn

Aragorn is a program for finding tRNAs and tmRNAs. Aragorn (version 1.1) can be run directly within DNA Master, although it is also accessible as a stand-alone program at:

http://130.235.46.10/ARAGORN/

The version of Aragorn available online is newer than the version embedded within DNA Master. It is **important to run the updated web-based version of Aragorn** (version 1.2.33.c.) in addition to the DNA Master version because it is better at determining the correct ends of tRNAs and because the version within DNA Master has a specific set of parameters that differ from the default. In addition, another tRNA predictor, tRNAscan-SE, is utilized to find additional tRNA predictions. Please refer to **Section 9.5** when you evaluate your tRNAs in your genome.

1.5 Setting Preferences

In general, setting preferences in DNA Master is a matter of opening the Preferences Window, making changes, and applying these changes. There are **five important preferences that you MUST set** before continuing with this guide. They are described in the next five subsections.

To get to the Preferences Window, select:

File → Preferences

You will see a dialog box with a series of tabs (Internet, Local Settings, …) each of which has another set of sub-tabs associated with it.

1.5.1 Set Default Translation Table & Template Insertion (Local Settings Tab)

Changing this setting ensures you are using the correct translation tables for phages. Select:

File → Preferences [Local Settings] [[New Features]]

- From the Default Translation Table dropdown menu, select '**Bacteria and Plant Plastid Code**'. Make sure that the boxes marked '**Add New Features to Documentation**', and '**Add New Features to Feature Table**' are both checked.
- Choose '**Insert template into notes during autoannotation'**. Add the following codes to the text box: **SSC: CP: SD: SCS: Gap: Blast: LO: ST: F: FS: ST:**. These codes are explained in **Section 9.6.**
- Click '**Apply**'. Note that the dialog box remains open.

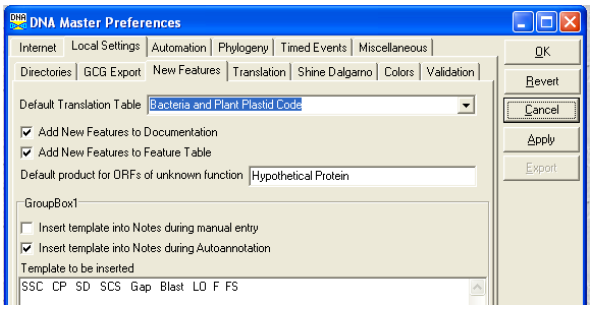


Figure 1.2

1.5.2 Set color preferences

You can select display colors for genes and tRNAs in various visual representations of your genome. The colors we recommend below are our preferences, and are used in most of the screenshots in this guide. You can select any colors you like, but note that if you use different colors, exported six-frame translations may not be properly viewable in Microsoft Word.

To set your colors to our recommended values, go to:

File → Preferences [Local Settings] [[Colors]]

Then set the values as shown below.

- Click on the colored box you want to change.
- A dialog box pops up with the color options.
- Click on the **color** of choice and then click **OK**.
- Continue to the next color.
- Don't forget to click '**Apply**' to save changes.

CDS Frame 1	**Yellow**	CDS Frame 4	**Gray**
CDS Frame 2	**Pink**	CDS Frame 5	**Light Green**
CDS Frame 3	**Light Blue**	CDS Frame 6	**Light Red**

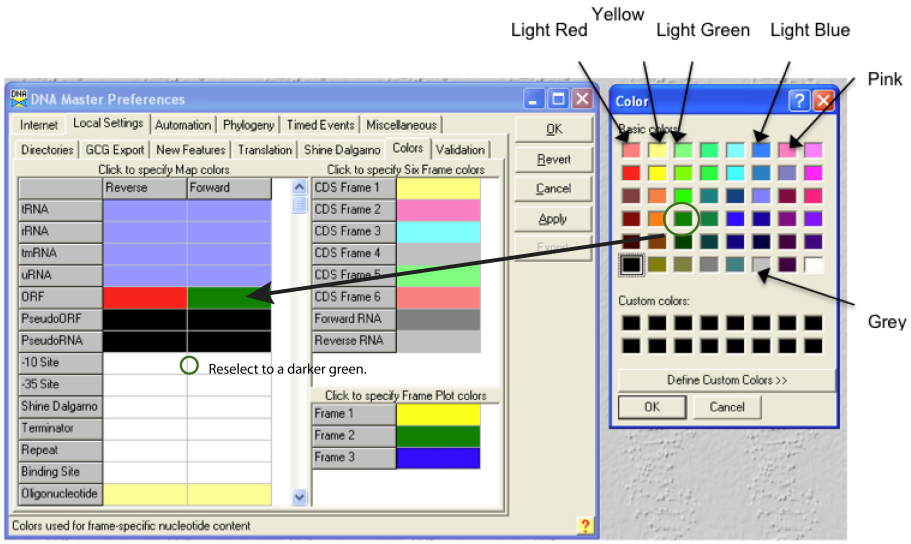


Figure 1.3

1.5.3 Set start codon choices

Because TTG is used as a translation initiation (start) codon in mycobacteriophage genomes – albeit rarely – you must make sure DNA Master recognizes it. To do so, go to:

File → Preferences [Local Settings] [[Translation]]

- All boxes must be checked, as shown in **Figure 1.4** below.
- Click '**Apply**'

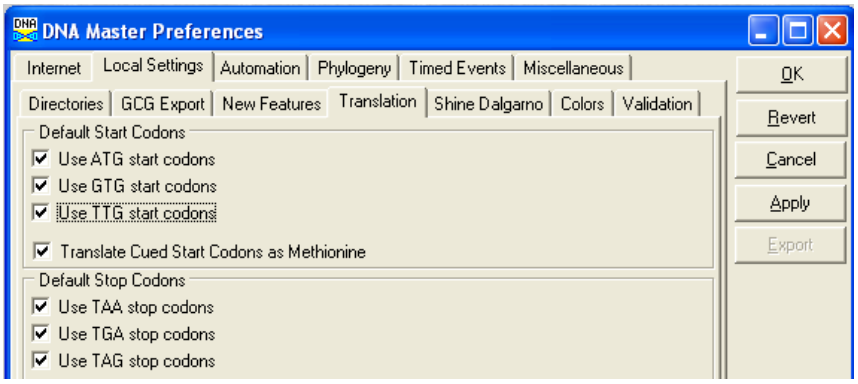


Figure 1.4

1.5.4 Set default values for BLAST searches

DNA Master can run batch BLAST searches and store the results for subsequent viewing. There are several settings relating to BLASTing inside DNA Master that may be helpful. Our suggestions are shown in **Figure 1.5**. Get to the BLAST menu by going to:

File → Preferences [Internet] [[Blast]]

- Set your preferences.
- Click '**Apply**' to save changes.
- Query timing may need to be adjusted depending on time of day that your send your BLAST request. See Section 4.5 for additional details

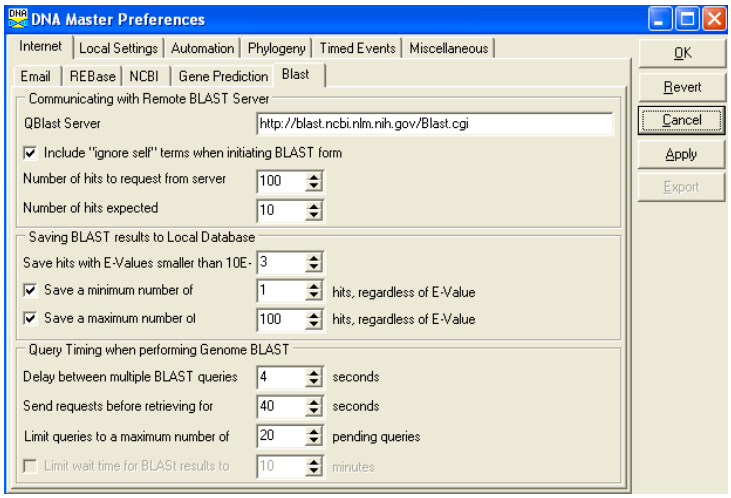


Figure 1.5

1.5.5 Choose a default location for saving files

DNA Master generates a number of files when it runs. It's good practice to create a dedicated DNA Master archiving folder, then direct DNA Master to use it. To do so, go to:

File → Preferences [Local Settings] [[Directories]]

- Click the '**Browse**' button next to the '**Archive to…**' field.
- Select your archiving folder, or create a new one.
- Click '**Apply**' to save.

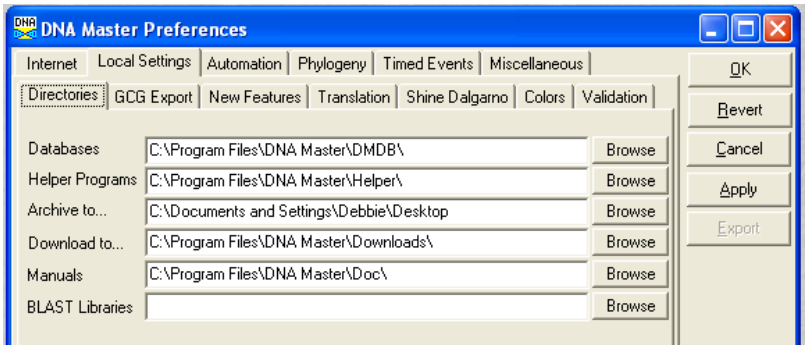


Figure 1.6

1.5.6 Finishing up your Preference settings

Once you have finished setting your DNA Master preferences:

- Click the '**OK**' button.
- Click '**Yes**' in the dialog box that asks if you want to save changes.

The Preferences Window will close.

1.6 Getting help

Help files and tutorials are available within DNA Master for many of its functions. Help is always available by clicking on the yellow ? button at the lower right corner of every window, or through the '**Help**' menu.

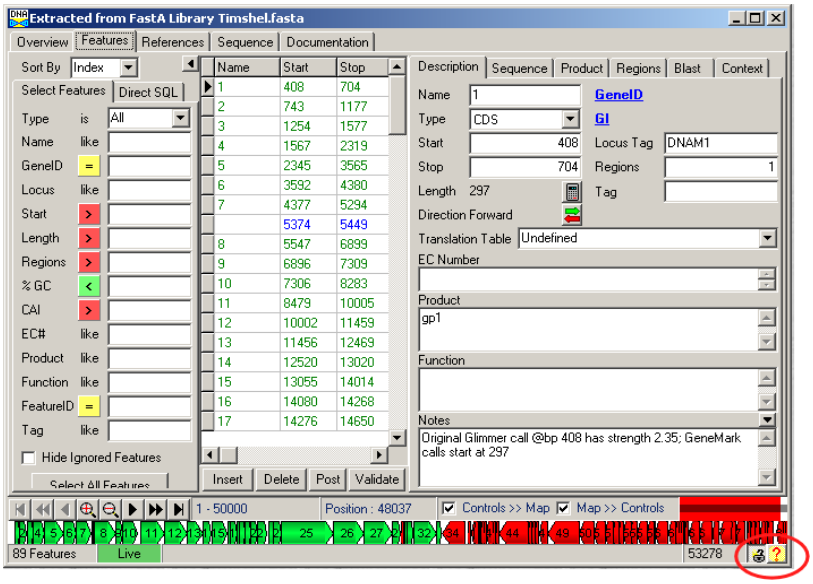


Figure 1.7

To get a sense of how the help files work, go to: **Help → Help**

- Read the 'Welcome to DNA Master' and the 'Getting Started Tutorial' sections.

1.7 Checking for updates

DNA Master is regularly updated, and with an internet connection it is easy to make sure your copy is up-to-date. Go to:

Help → Update DNA Master

- If a new version is available, it will update the program, and a dialog box will appear when completed. Please note that you must have an active internet connection to do this!
- When the update is complete, close and restart the program.
- As of the time of writing (November 2014), the most up-to-date version of DNA Master is Version 5.22.19 Build 2448, dated 13 June 2014. You can find your current version by going to: **Help → About**

1.8 Event Manager

The Event Manager (Tools -> Event Manage) is a useful tool when you receive error messages in DNA Master. There is far more information recorded here than what is provided on screen.

2 Provisional Cluster assignment of your phage

2.1 Overview

All sequenced mycobacteriophage genomes have been compared to one another, and based on these comparisons they have been grouped into **clusters** of related phages. Some of these clusters are small (Cluster V currently has only two members), whereas others are quite large (Cluster A has 259 members). Some clusters are further divided into **subclusters**; for example, Cluster B's genomes are currently divided into five subclusters: B1, B2, B3, B4, and B5. There are also some phages (nine currently) who have no close relatives, and therefore are classified as **Singletons**. Up-to-date statistics are available at:

http://phagesdb.org/clusters/

Your phage's final cluster designation depends on a variety of analyses, as described in:

Hatfull *et al*., (2010) Comparative genomic analysis of sixty mycobacteriophage genomes: Genome clustering, gene acquisition and gene size. *J Mol Biol*. **397**, 119-143.

In the meantime, however, it is helpful to make a provisional cluster assignment for your phage. This can be done using just a completed genome sequence, before any annotation has taken place because clustered phages usually share a span of 50% or more recognizable nucleotide similarity across their genomes.

Performing a nucleotide BLAST search of your phage sequence against a database of mycobacteriophage genomes (at phagesDB.org) provides a simple and quick approach to making a provisional cluster assignment. The assignments made at the completion of a genome sequence are based on this BLAST alignment. Further analysis made be necessary for some genomes.

Note: It is not reliable to denote cluster designations without a complete genomic sequence available. Inferences can be made about cluster membership through a variety of ways however these should be included in isolation notes and not used to label a phage as a member of a particular cluster.

2.2 BLASTing your sequence against the mycobacteriophage database

To BLAST your genome on phagesdb.org (nucleotide BLAST):

- Go to http://phagesdb.org/phages/
- Locate your phage in the phage list, then click to open its detail page.
- Click on the green "**Locally BLAST this genome**" button.
- It will open a page that looks like the one in **Figure 2.1**.

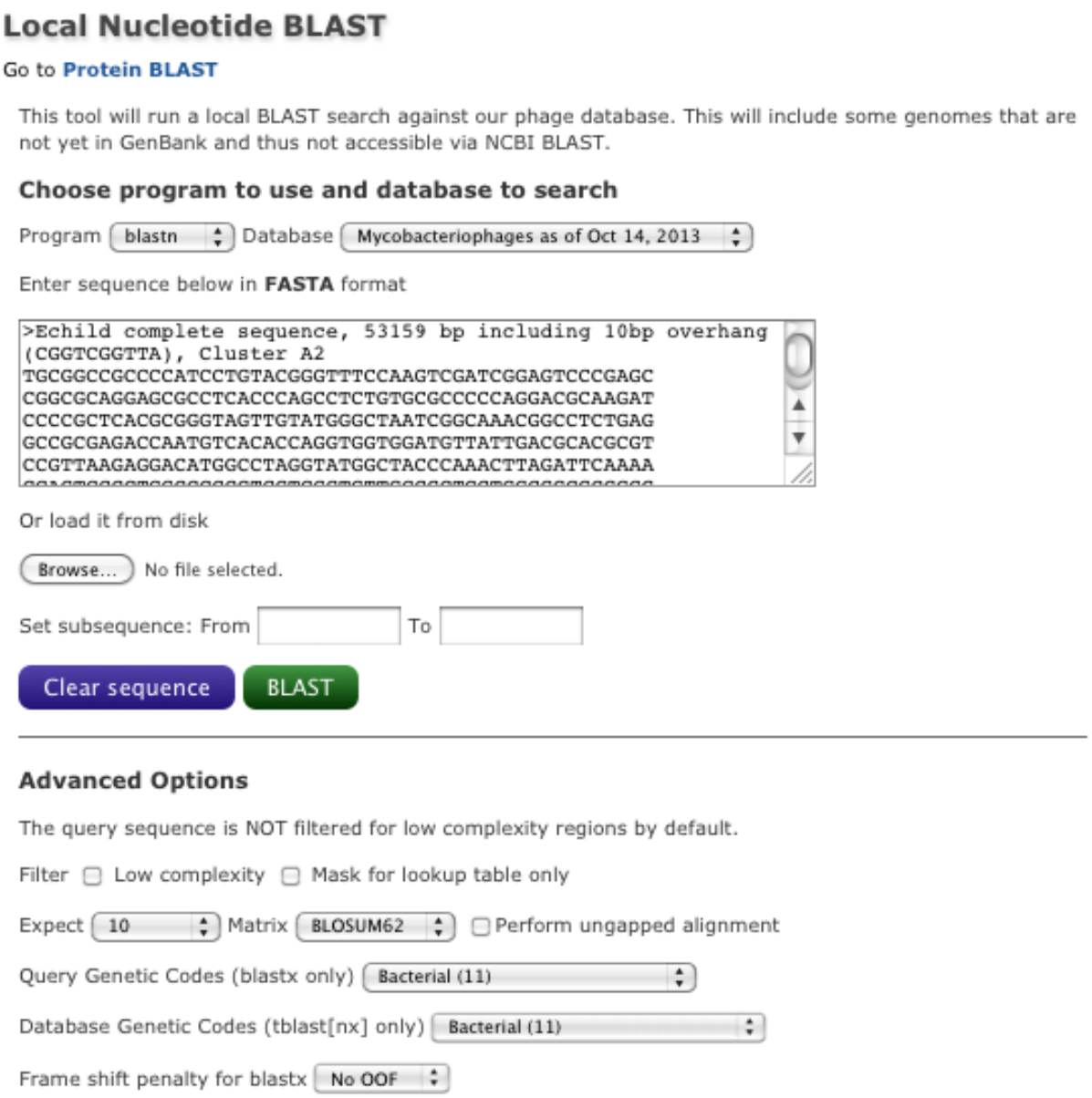


Figure 2.1

- The defaults are set so that the program will run **blastn** (i.e. a nucleotide search against a nucleotide database) against a database of previously sequenced mycobacteriophage genomes (e.g., Mycophages as of 6.01.11).
- Click on the '**BLAST!**' button. It is just above the gray dividing bar at the center of **Figure 2.1** above.

A new page will open showing the results of the BLAST search, as shown in **Figure 2.2** below.

Your query is represented by a black bar underneath "Color Key for Alignment Scores". Subject sequences from the database that align well to your query sequence are represented by colored bars beneath the black bar. The length and location of the subject bars indicates the portion(s) of the query sequence the subject sequences match. The quality of each alignment is shown by color, with the best matches colored red.

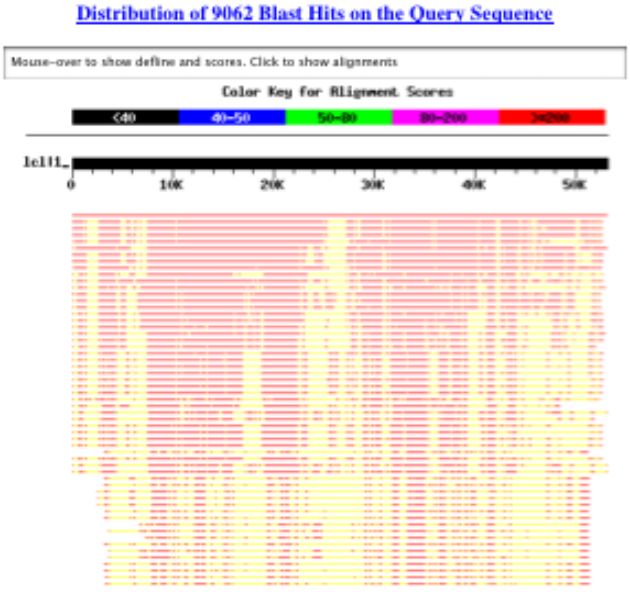
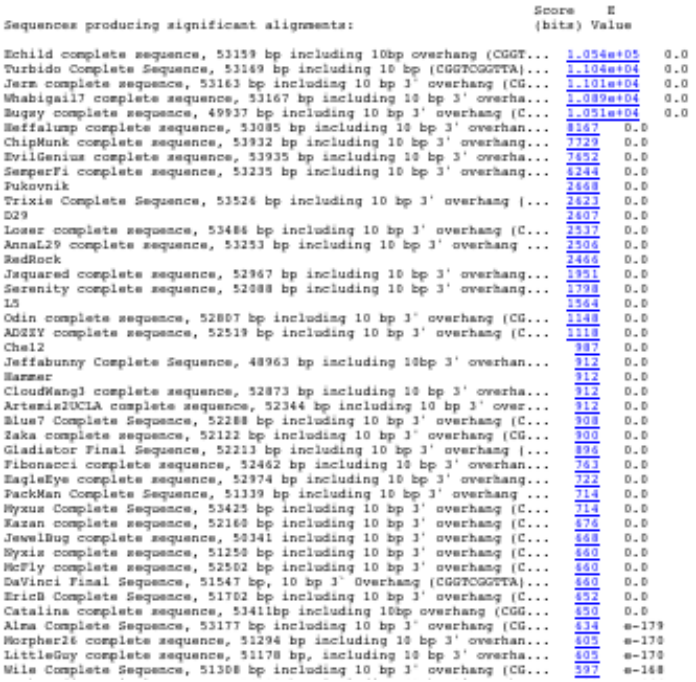


Figure 2.2

To see which subject sequences your query has aligned to, simply mouseover any of the colored bars, and the subject's name will appear in the box above the "Color Key for Alignment Scores". Then, either scroll down or click on one of the lines to get the names of subject sequences that have the best alignments to your query sequence, listed in order from best match to worst match (see below). After each subject sequence name is the raw score of the alignment to your query sequence (higher is a better alignment), and the E value (lower is a better alignment).



Sequences producing significant alignments:	Score (bits)	E Value
Echild complete sequence, 53159 bp including 10bp overhang (CGGT...	1.054e+05	0.0
Turbido Complete Sequence, 53169 bp including 10 bp (CGGTCGGTTA)...	1.104e+04	0.0
Jerm complete sequence, 53163 bp including 10 bp 3' overhang (CG...	1.101e+04	0.0
Whabigail7 complete sequence, 53167 bp including 10 bp 3' overha...	1.089e+04	0.0
Bugsy complete sequence, 49937 bp including 10 bp 3' overhang (C...	1.051e+04	0.0
Heffalump complete sequence, 53085 bp including 10 bp 3' overhan...	8167	0.0
ChipMunk complete sequence, 53932 bp including 10 bp 3' overhang...	7729	0.0
EvilGenius complete sequence, 53935 bp including 10 bp 3' overha...	7852	0.0
SemperFi complete sequence, 53235 bp including 10 bp 3' overhang...	6244	0.0
Pukovnik	2468	0.0
Trixie Complete Sequence, 53526 bp including 10 bp 3' overhang (...	2623	0.0
D29	2607	0.0
Loser complete sequence, 53486 bp including 10 bp 3' overhang (C...	2537	0.0
AnnaL29 complete sequence, 53253 bp including 10 bp 3' overhang ...	2506	0.0
RedRock	2466	0.0
Jsquared complete sequence, 52967 bp including 10 bp 3' overhang...	1951	0.0
Serenity complete sequence, 52088 bp including 10 bp 3' overhang...	1798	0.0
L5	1564	0.0
Odin complete sequence, 52807 bp including 10 bp 3' overhang (CG...	1148	0.0
ADZZY complete sequence, 52519 bp including 10 bp 3' overhang (C...	1118	0.0
Che12	987	0.0
Jeffabunny Complete Sequence, 48963 bp including 10bp 3' overhan...	912	0.0
Hammer	912	0.0
CloudWang1 complete sequence, 52873 bp including 10 bp 3' overha...	912	0.0
Artemis2UCLA complete sequence, 52344 bp including 10 bp 3' over...	912	0.0
Blue7 Complete Sequence, 52288 bp including 10 bp 3' overhang (C...	908	0.0
Zaka complete sequence, 52122 bp including 10 bp 3' overhang (CG...	900	0.0
Gladiator Final Sequence, 52213 bp including 10 bp 3' overhang (...	896	0.0
Fibonacci complete sequence, 52462 bp including 10 bp 3' overhan...	763	0.0
EagleEye complete sequence, 52974 bp including 10 bp 3' overhang...	722	0.0
PackMan Complete Sequence, 51339 bp including 10 bp 3' overhang ...	714	0.0
Myxus Complete Sequence, 53425 bp including 10 bp 3' overhang (C...	714	0.0
Kazan complete sequence, 52160 bp including 10 bp 3' overhang (C...	676	0.0
JewelBug complete sequence, 50341 bp including 10 bp 3' overhang (C...	668	0.0
Myxis complete sequence, 51250 bp including 10 bp 3' overhang (C...	660	0.0
McFly complete sequence, 52502 bp including 10 bp 3' overhang (C...	660	0.0
DaVinci Final Sequence, 51547 bp, 10 bp 3' Overhang (CGGTCGGTTA)...	660	0.0
EricB Complete Sequence, 51702 bp including 10 bp 3' overhang (C...	652	0.0
Catalina complete sequence, 53411bp including 10bp overhang (CGG...	650	0.0
Alma Complete Sequence, 53177 bp including 10 bp 3' overhang (CG...	634	e-179
Morpher26 complete sequence, 51294 bp including 10 bp 3' overhan...	605	e-170
LittleGuy complete sequence, 51178 bp, including 10 bp 3' overha...	605	e-170
Wils Complete Sequence, 51308 bp including 10 bp 3' overhang (CG...	597	e-168

Figure 2.3

Scroll down further (or click on the blue raw score number) to get the nucleotide alignment of your query sequence (top) to each subject sequence (bottom). The numbers on the sides of the sequences indicate the nucleotide coordinates within each sequence. Identical nucleotides are connected with vertical lines and smaller gaps in the alignment are shown by horizontal dashes.

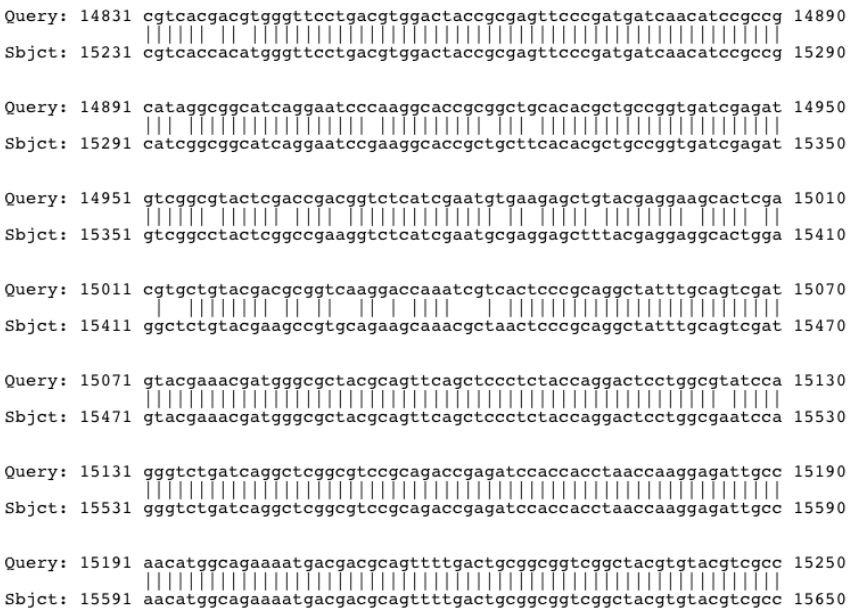


Figure 2.4

2.3 Cluster assignment

You should now be able to make a provisional cluster assignment. If one of your subject matches is a red line extending over at least 50% of the genome, then it is likely that your phage belongs in the same cluster as that subject. If the cluster is divided into subclusters, then a long but interrupted red line likely indicates that it is similar to a particular subcluster.

We'll use a case study—the phage Adephagia—to demonstrate how to assign a provisional cluster to your phage. **Figure 2.5** shows the output of a BLAST search with the Adephagia genome as the query.

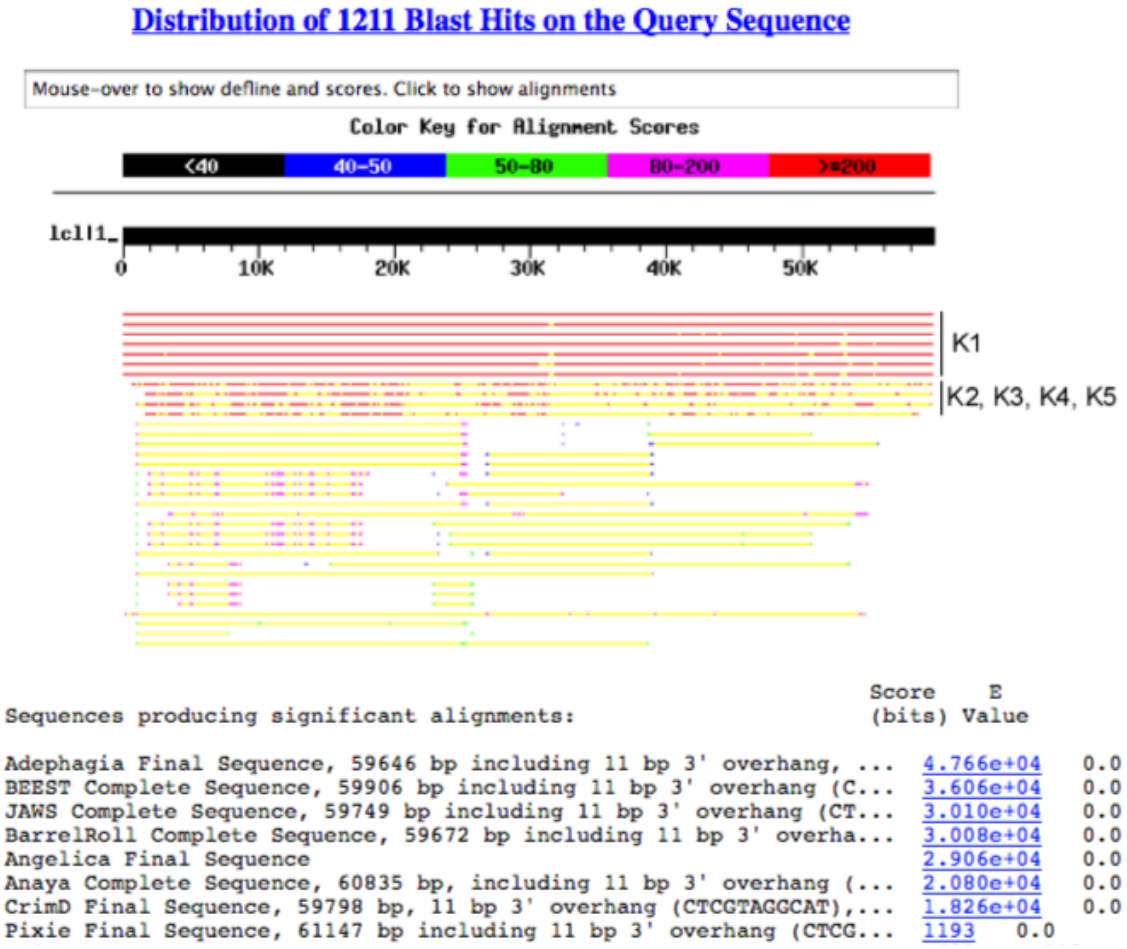


Figure 2.5

Adephagia's best hit is to itself. After that, there are six heavy red lines that indicate very similar genomes to Adephagia's. Scrolling down to the "Sequences producing significant alignments" section, we can see that these red lines correspond to the genomes of BEEST, JAWS, BarrelRoll, Angelica, Anaya, and CrimD. Using phagesdb.org, we can then look up the cluster assignments of these six phages. All six, it turns out, are members of Cluster K, and Subcluster K1.

There are four more genomes that appear to have significant similarity to Adephagia, though the matches are less solid and cover less of the query sequence. These more tattered-looking red lines correspond to Pixie, TM4, Larva, and Fionnbharth. Using phagesdb.org, we can see that these are all member of Cluster K, though they belong to Subclusters K2-K5, not K1.

Therefore, we can provisionally determine that Adephagia is a member of **Cluster K** and **Subcluster K1**.

NOTE: Though the example above may seem clear-cut, cluster assignment will not always be so simple. If it's not, don't be concerned. You may have found a new singleton phage, or a phage that will lead to a new subcluster being created. The main point of doing this now is so that you have an idea of which phages are most closely related to the one you are annotating. These closely related phages can be very useful guides as you go through the annotation process.

3 Importing your phage genome sequence into DNA Master

3.1 Overview

Now that you have a sense of your software and an overview of your phage genome, you are ready to move onto the really exciting stuff! The first thing you need to do is to download your phage's genome sequence, then import it into DNA Master.

3.2 Where do I get my phage genome sequence from?

Sequencing a phage genome involves two parts: Shotgun Sequencing and Finishing (aka Polishing). The second part, **Finishing**, involves generating targeted reads to fix weak areas, determining the type and/or sequence of genome ends, and orienting a genome to match convention. When performing annotations, you **must always use a Finished sequence file**, or your annotation work may have to be redone.

Fortunately, **phagesdb.org** only posts Finished sequence files, so be sure to get your sequence from phagesdb.org. Though you may have access to preliminary, un-Finished files from other sources, **the phagesdb.org site should be the only source for sequence when beginning annotation.**

A NOTE ON FILE TYPES

DNA, RNA, and protein sequence files are often saved in **fasta** format. This is the standard format required by many bioinformatics programs, including BLAST. Fasta files are simply text files where:

1. The first line begins with ">" and contains information about the sequence
2. Subsequent lines contain the sequence itself

For example, the first few lines of a phage genome sequence fasta file may look like:

```
> Giles Complete Genome Sequence, 53746 bp
GGCAGACTTTTTTTTGCGCGGCGGCCTGCGCGCGCGGCCCCGCCCGCCCC
GCCGGGTCGGAGGCGGCCGAATGACGCCACCTCGGGCCGCGGTGGCCGAC
ACGCCGGATACGCCCGCAGAGGGCAAATCAGGGGCCAAAACGCGGGCCAA
```

A few things to keep in mind:

- Fasta files can be opened with any text editor.
- A file does not need to have the extension **.fasta** to be in fasta format. For example, if you rename Giles.fasta to Giles.txt, the file will still be fasta-formatted.
- Sequence files from phagesdb.org are in fasta format and have a .fasta extension.

To download your genome sequence as a fasta file, go to:

- http://phagesdb.org/phages/
- Scroll down to find your phage and click its name to open its detail page.
- Scroll down to the section titled 'Sequencing Information'.
- Click on the '**Download fasta file** ' link, and save the file to a known location.

IMPORTANT NOTES:

- If you can't find the downloaded file, simply search your computer for a file named YourPhageName.fasta.
- If you are using a Windows emulator on a Mac (and use your internet browser on the Mac side to get the fasta file), then you should either copy the fasta file from the Mac side to the Windows side, or alternatively set up your emulator preferences so that it can directly read files from the Mac side from a shared folder.
- If for some reason you're using a sequence file from a location other than phagesdb.org, be mindful that there are two possible orientations for a genome, and that yours needs to conform to the standard convention (the virion structural genes on the left, transcribed rightwards). If you determine that a sequence needs to be reverse-complemented, instructions are provided at the end of this section for doing so.

3.3 *Importing your DNA sequence into DNA Master*

You are now ready to import your fasta file into DNA Master. Open DNA Master, then go to:

File → Open → FastA Multiple Sequence File

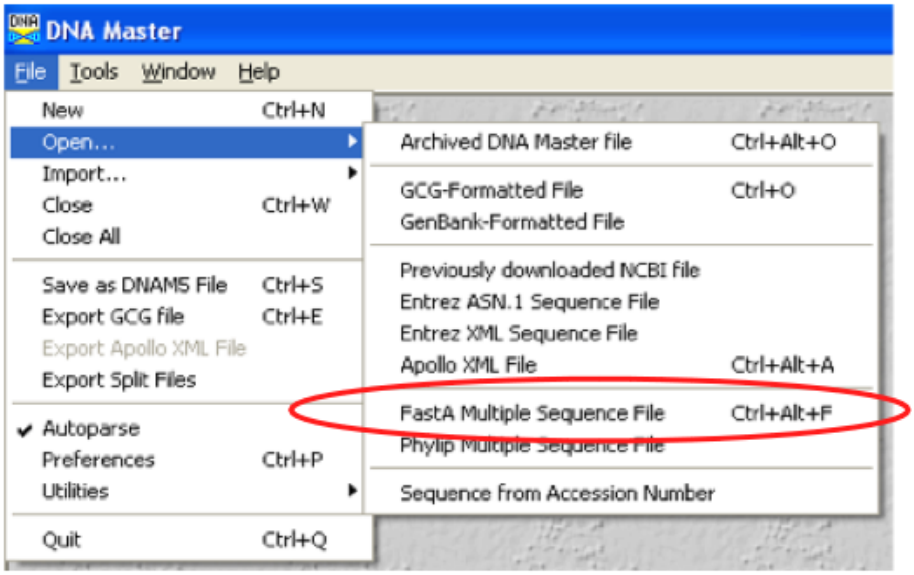


Figure 3.1

- Browse to the correct folder and select your fasta file.

- A window like the one shown in **Figure 3.2** appears.

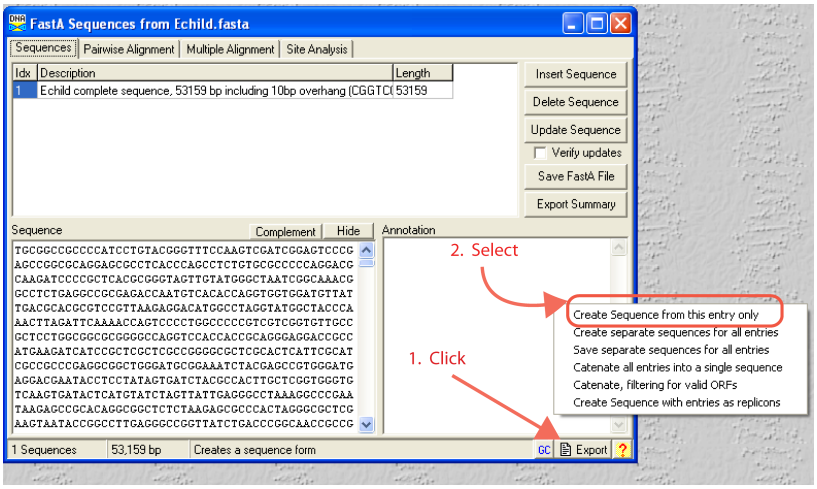


Figure 3.2

- Click on the Export button in the lower right hand corner (1).
- From the menu that opens, select '**Create Sequence from this entry only**' (2).
- A new window titled '**Extracted from FastA library YourPhage.fasta**' will open within DNA Master.
- We recommend that you now save the file with a new name, for example Sheen.dnam5. Once the file is in this format, you will always open an 'archived DNA Master file.

Let's take a moment to look at some of the new views that are available.

- There are five tabs in the new window: **[Overview]**, **[Features]**, **[References]**, **[Sequence]**, and **[Documentation]**.
- Select the **[Overview]** tab if it's not already selected. Your window should look similar to the one in **Figure 3.3**.

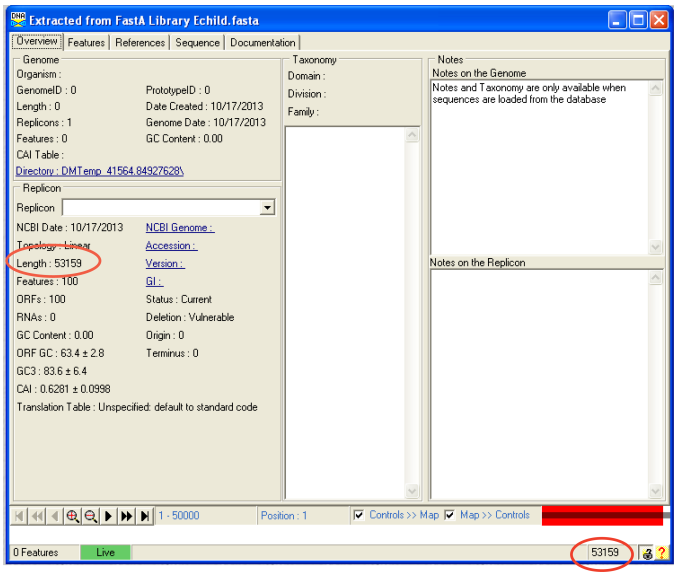


Figure 3.3

- Check the sequence length (shown in the red circles in **Figure 3.3**) and verify that it matches the published sequence length on your phage's detail page on phagesdb.org. If there is a discrepancy, restart the program and try importing again, or re-download your sequence file from phagesdb.org.
- Select the **[Sequence]** tab. This tab displays the DNA sequence of your phage. You can click and drag to select part of the sequence, whereupon DNA Master will display the coordinates and length of the selected portion near the top of the window, as in **Figure 3.4**.

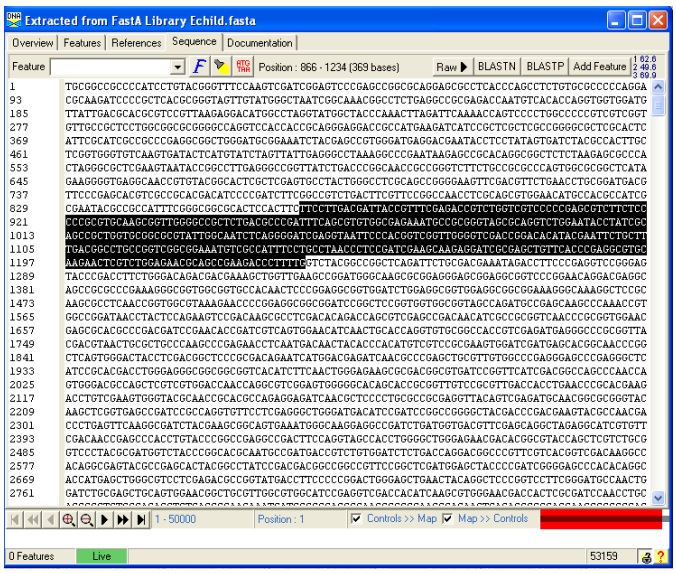


Figure 3.4

- Until you run an automated annotation in the next section, the tabs for **[Features]**, **[References]**, and **[Documentation]** are largely empty.

Congratulations! You have now imported your phage sequence into DNA Master and are ready to run an Auto-Annotation.

3.4 Reverse-complementing your sequence

To re-emphasize, if you download your genome sequence from phagesdb.org, it will **NOT** need to be reverse-complemented. If you need to reverse-complement a sequence from a different source to match conventions, you can do so easily within DNA Master. To reverse-complement a sequence:

- Go to the **[Sequence]** tab.
- **Make sure that no segment of the sequence is selected** (otherwise you will only flip that part—a big mess). If in doubt, just click somewhere within the sequence, but without selecting anything.
- Select: **DNA → Convert → Complement**

- A dialog box will open that asks if you want to convert XXXXX bp to 5′ → 3′. Click '**Yes**'. (XXXXX should be your full genome length)
- Select: **File → Save as ,** then save your reverse-complemented file with a new name.

4 Performing and viewing a rapid automated annotation of your genome

4.1 Overview

DNA Master has an **Auto-Annotate** function that provides quick and simple identification of genes within your phage genome. It works by running Glimmer, GeneMark, and Aragorn, then combining the outputs from these programs to arrive at consensus gene calls. The consensus output is used to populate DNA Master's Documentation and Feature Table sections.

Generally, this auto-annotation will identify 90% or more of the genes accurately, but the careful refinement that you will perform in **Section 8** will be essential for obtaining the best possible annotation that will be ready for GenBank submission.

4.2 Running Auto-Annotate

- As shown in **Figure 4.1**, go to:

Genome → Annotation → Auto-Annotate

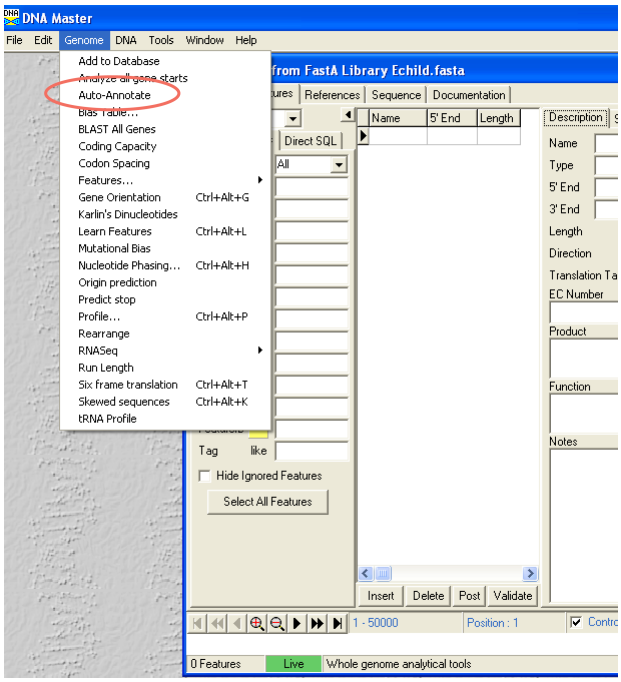


Figure 4.1

- An Auto-Annotate dialog box will open, with 4 sub-windows to configure. We recommend that you use the settings shown in **Figure 4.2**.

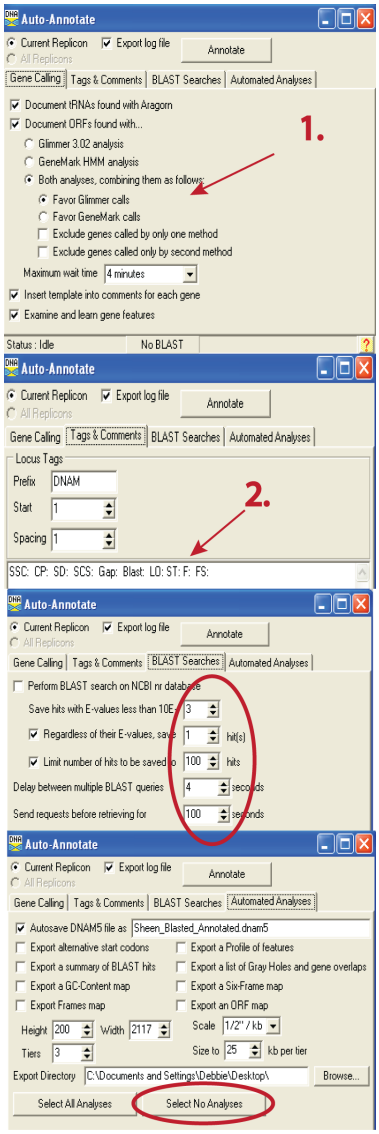


Figure 4.2

1. In the **Gene Calling** window: Choose both analyses, favoring Glimmer.'
2. Include the following Template in **Tags & Comments** window: '**SSC: CP: SD: SCS: Gap: Blast: LO: ST: F: FS':** (See **Section 9.6** for details) if you entered this in the Preferences Settings, this list will automatically appear.
3. Unclick Perform Blast searches in the **BLAST searches** window.
4. Select No analyses in the **Automated Analyses** window.
5. Click the '**Annotate**' button to launch the automated annotation. (Click '**Yes**' when prompted to "Erase features?")

The auto-annotation (without BLAST) takes only minutes. As the auto-annotation proceeds, the status of the process will be displayed at the lower left corner of the auto-annotate window. You will see Predict genes, predict tRNAs. Parsing, …) When complete, an auto-annotation log will be generated. Review that what you requested was actually done. Then close that window and you are ready to review the genome.

SOME NOTES ON AUTO-ANNOTATE OPTIONS

- One key Auto-Annotate option is the '**Perform BLAST searches on nr database**' checkbox. When checked, this option will BLASTP the protein product of each gene Auto-Annotate finds, then save the results for viewing later—a powerful tool, and recommended if you have the time. However, performing that many BLAST searches often takes more than 45 minutes, during which DNA Master will be inaccessible. If you'd like to move on to further steps quickly, uncheck this box and Auto-Annotate will run in fewer than five minutes.

  See **Section 4.5** for how to BLAST genes at a later time.

- In the Gene Calling pane, we prefer to use the default option of using '**Both analyses**' (Glimmer and GeneMark), with '**Favor Glimmer Calls**' selected. Often, the two programs' gene calls differ only in the location of the start codon, so it doesn't really matter. If desired, you can try modifying options to see their effects on the resulting gene calls. Auto-Annotate runs quickly enough to experiment!

  When there is a conflict between Glimmer and GeneMark calls, both calls will be reported in the gene's Notes. If the two programs agree on a gene, the Notes will list the favored program's call only.

- The checkbox to '**Export a Six-Frame map**' produces a translation of the sequence in all six frames, a useful asset for annotation. See Section 5 for generating maps and translations. at a later time.

4.3 Saving your file

As with any program, it is important to **save your file often** to protect changes you've made from being lost. This can be done by going to:

File → Save as DNAM5 file

Choose a new file name if you wish to keep both previous and current versions. This is a way to keep backups of work you've done. To avoid confusion about which file is the current version, it is helpful to establish systematic naming conventions when saving files. Remember to recreate your Documentation prior to saving.

If you are prompted to save the file before closing, always save it with a new name. Do not re-write the last version. (When prompted to "Save before closing?" after you have saved it as a newly named file, your response is "No".)

The input file format was a fasta, but now it is a DNA Master file. The format of the file will now be [your phageName].dnam5. Therefore, when you save and re-open the file you will open as an "Archived DNA Master file".

4.4 *Looking at the output of your automated annotation*

Once the Auto-Annotate function has run, it will return you to your main phage window. Under the **[Overview]** tab, however, you will see some immediate differences.

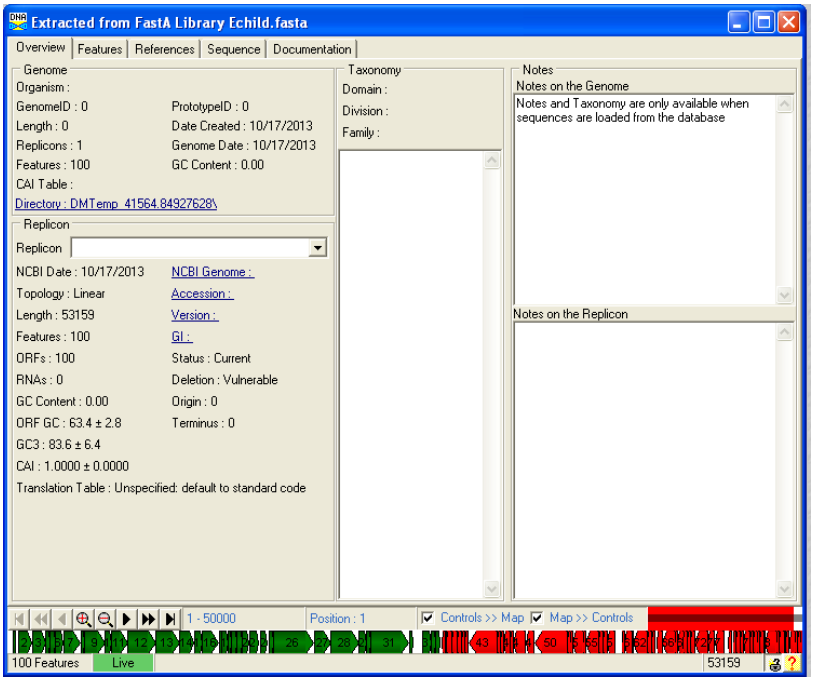


Figure 4.3

For example, note that there is a map showing the predicted genes at the bottom of the window. Genes transcribed leftwards and rightwards are shown in different colors depending on how you have set your DNA Master preferences (**Section 1.6.2**; green and red in **Figure 4.3**).

This map is dynamic and can be manipulated as follows:

- Roll your mouse over the map. You will see the number changing in the box above it labeled '**Position**'. This reports the coordinate in the genome where your mouse is pointing.
- Click on the  button to zoom in and the  button to zoom out.
- Click on the left and right arrows to move  a little each way,  a lot each way, or  to the extreme left or right ends.

4.4.1 Viewing the documentation

Auto-Annotate writes its output to the **Documentation**. Though you will generally work in the **[Features]** tab, it is useful to be familiar with this underlying Documentation. Click on the **[Documentation]** tab to take a look.

You will see that DNA Master has populated the Documentation with the consensus outputs from Glimmer, GeneMark, and Aragorn. In the example shown in **Figure 4.4**, the first line says "CDS (330-443)". This means the first feature is a protein-coding sequence (CDS) transcribed left to right and located at coordinates 330 – 443.

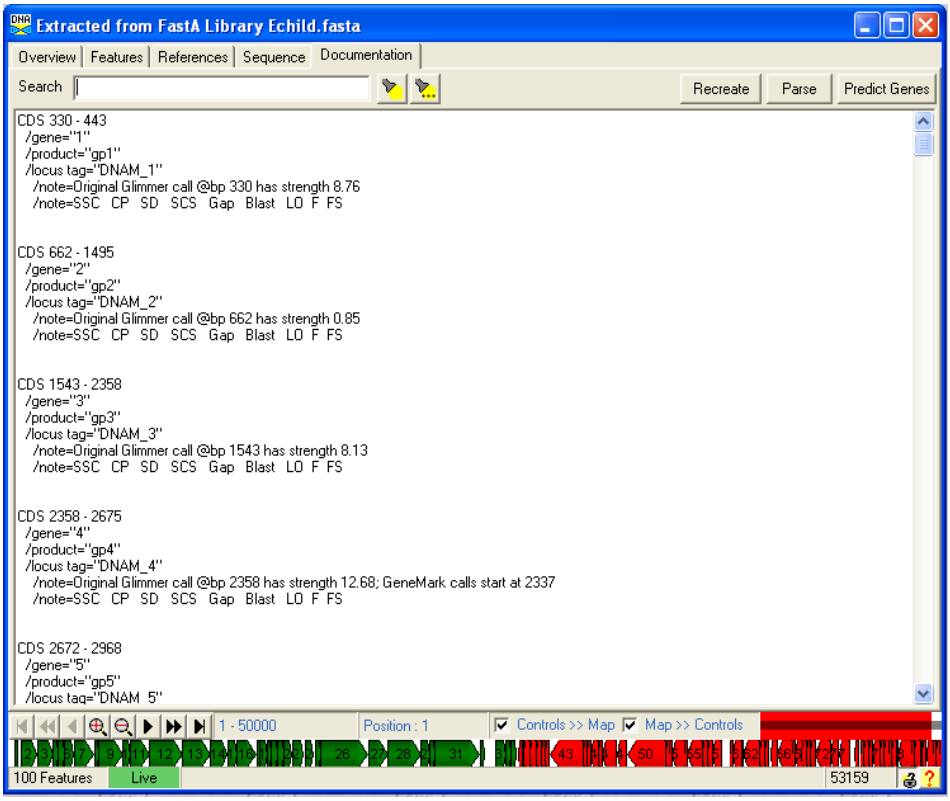


Figure 4.4

Additional data for each feature are shown in the indented lines that follow. For example, the first feature has a gene name of "**1**", a protein product named "**gp1**", a locus tag of "**DNAM1**", and a note about where Glimmer called the start position. The Notes also contain the template that you entered in Preferences (**Section 1.6**).

The data contained in the Documentation are also viewable in the Features Table (see below).

4.4.2 Viewing features in the Feature Table

The Documentation that you viewed above has been automatically **Parsed** by DNA Master into the **Feature Table**. Click on the **[Features]** tab to view the Features Table (**Figure 4.5**).

Note that the Documentation window is a text file. You can copy and paste its entire content. What you see is what you get. The same is not true for the Feature Window. When you are in the Feature Window, you are looking at the contents of a database that is being constructed about each feature of this project. You cannot Copy & Paste this window as you see it.

Figure 4.5 demonstrates the first modification you will want to make to this window, namely "Widen the Feature Table". In the current form, the Feature Table displays the Name, 5′ End, and Length of each feature. In order to also display the 3′ End, right click on the heading of the table (1) and select "Widen Feature Table" (2). The list will now display each gene's **Tag** (Locus Tag), **Name, 5′ End, 3′ End**, and gene **Length**. You can select any gene by clicking on it. Gene "**1**" is selected in the example below, as indicated by the small black triangle next to it.

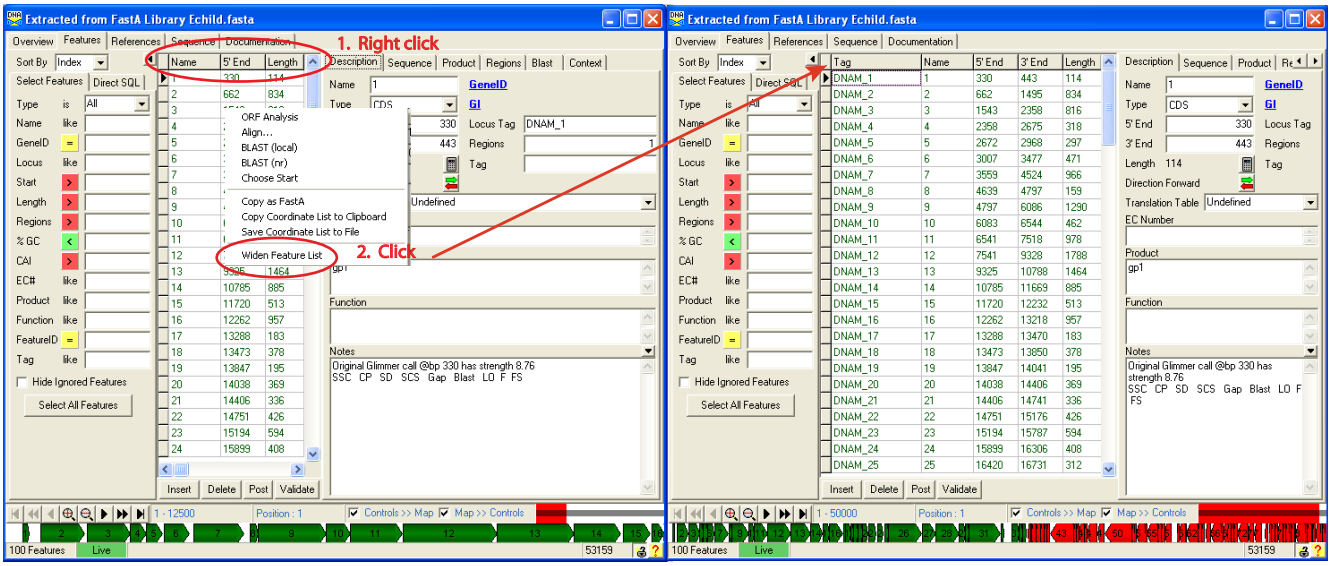


Figure 4.5

If you look to the right, you will see six sub-tabs named **[[Description]]**, **[[Sequence]]**, **[[Product]]**, **[[Regions]]**, **[[Blast]]**, and **[[Context]]**.

The **[[Description]]** sub-tab is shown by default and contains basic information about the gene that you'll recognize from the documentation, including gene name, coordinates, product name, and notes.

DNA Master imports the data of the best Glimmer and GeneMark predictions. It reports it in the order in of your auto-annotation. If you ran both analyses and favored Glimmer, the notes will reflect that in the following ways.

The **Notes** for gene 1, shown above, indicate that Glimmer called the start at position 330. There is no mention of GeneMark in these notes, which means that GeneMark's gene call agreed with Glimmer's gene call. If the two programs do not agree, this will be mentioned in the Notes as shown in **Figure 4.6**. Check out genes 61 and 63.

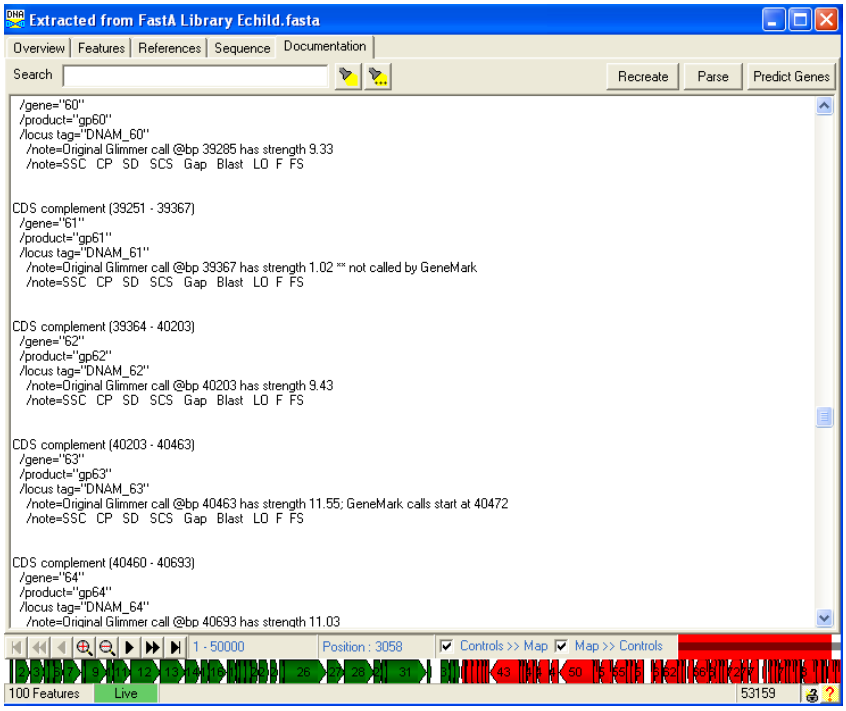


Figure 4.6

In the next example, gene 61 was predicted by Glimmer, but was "not called by GeneMark".

For gene 63, the assigned start is 40203 as called by Glimmer, but there is a note that "GeneMark calls start at 40472".

There is one more alternative to this notation. The notes can say GeneMark call @bp XXXX. This notation means that this gene was not called by Glimmer.

Your refinement of your annotation in **Section 8** will focus substantially on evaluating the predictions made by Glimmer and GeneMark. You will be resolving any ambiguities that have arisen and adding or deleting genes that were missed or errantly called by these programs.

You don't need them just yet, but you can see that there are also buttons (at the bottom of the central box middle) that will let you either '**Insert**' or '**Delete**' features. And eventually the '**Validate**' button will help you assess whether all your gene calls make sense.

4.4.3 Viewing the sequence in the Sequence tab

Click on the **[Sequence]** tab.

You will see the sequence appear as before, but now you can use the '**Feature**' dropdown menu at the top left. When you click on this menu, a list appears that shows each gene and whether it is transcribed leftwards (R, for reverse) or rightwards (F for forward).

You can scroll down and select any of these and it will then select and highlight the corresponding part of the DNA sequence. This can be a very useful feature for examining specific parts of the genome.

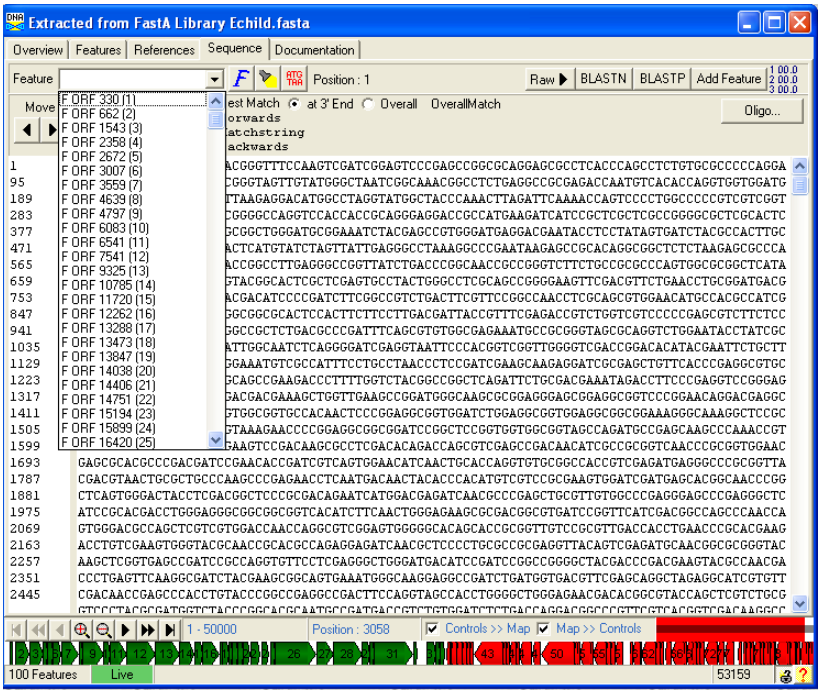


Figure 4.7

4.4.4 Viewing ORFs in the Frames window

The Frames window is an especially important one for determining and assessing start site choices. To open the Frames window (we use Angelica in the example below) select:

DNA → Frames

A window will open that has a graphical representation of the six possible translation reading frames, with each row representing one reading frame. Full-row-height vertical lines represent in-frame stop codons, and half-row-height vertical lines are possible start codons. At the lower left in the window is a box displaying the nucleotide coordinate corresponding to the position of your pointer as you mouse over the display. There are also buttons that allow you to scroll through your genome and zoom in and out.

At the lower right corner of the Frames window, there are seven additional buttons. Click on the button labeled '**ORFs**' (red circle in **Figure 4.8**).

This will highlight all the features currently in your feature table as shown in the screenshot below. Genes in forward reading frames are green, those in reverse reading frames are red, and tRNAs are blue. Note that the genes are numbered in this format also.

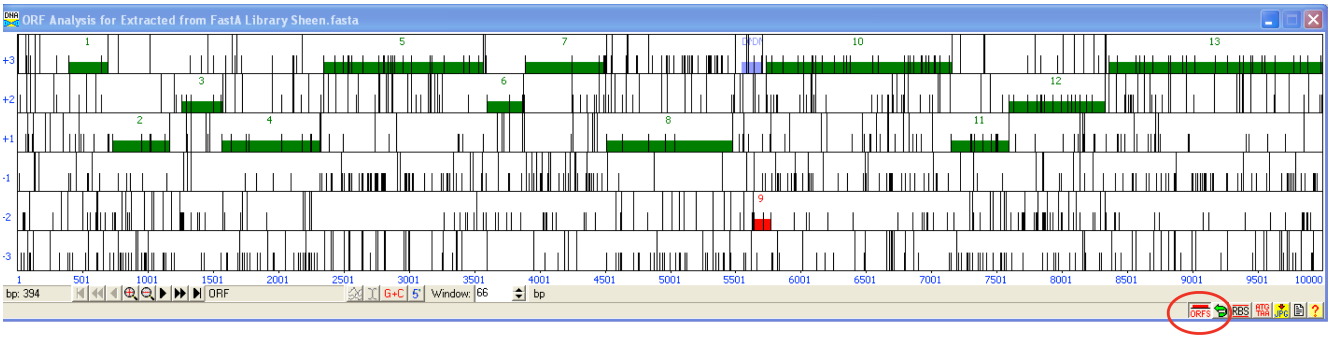


Figure 4.8

This next screen shot of the Frames window has been modified to expand the frames. This is done by clicking on the  button to zoom in. In order to a select a gene (gene 6 in this example) click in the ORF (box) that contains the highlighted gene (Figure 4.9).

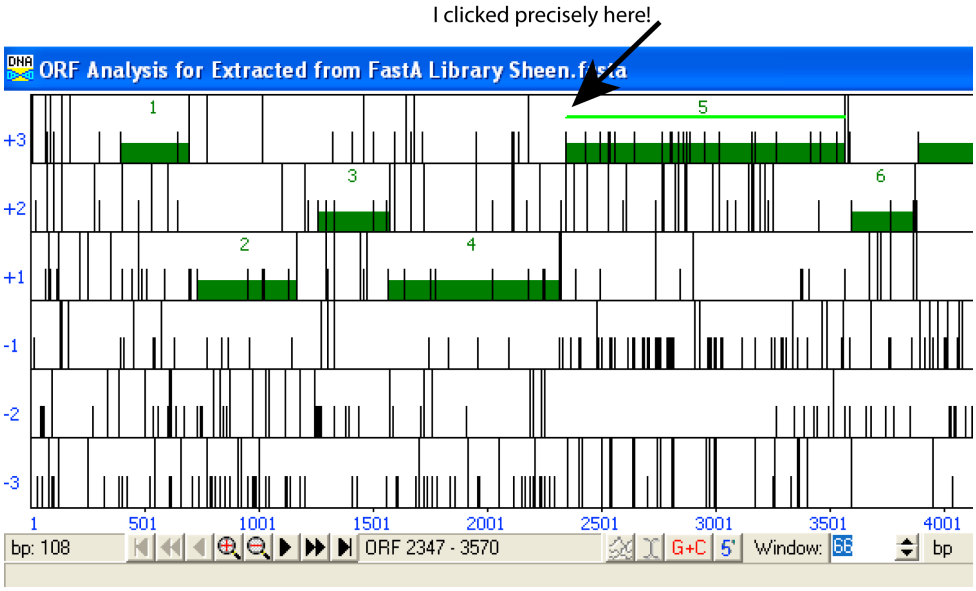


Figure 4.9

A thin, horizontal green line will appear that extends from the nearest upstream start codon to the next downstream stop codon. Now click on the '**RBS**' (ribosomal binding site) button in the bottom right corner of the Frames window (**Figure 4.10**).

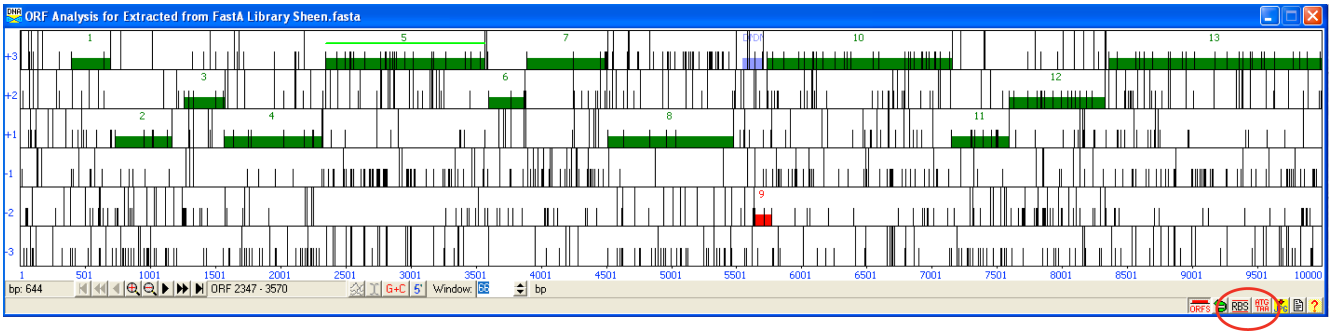
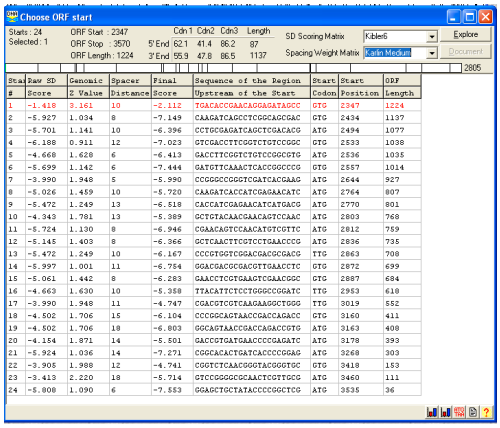


Figure 4.10

Another window titled "**Choose ORF start**" will appear, shown in **Figure 4.11**.



Sta #	Raw SD Score	Genomic Z Value	Spacer Distance	Final Score	Sequence of the Region Upstream of the Start	Start Codon	Start Position	ORF Length
1	-1.418	3.161	10	-2.112	TGACACCGAACAGGAGATAGCC	GTG	2347	1224
2	-5.927	1.034	8	-7.149	CAAGATCAGCCTCGGCAGCGAC	GTG	2434	1137
3	-5.701	1.141	10	-6.396	CCTGCGAGATCAGCTCGACACG	ATG	2494	1077
4	-6.188	0.911	12	-7.023	GTCGACCTTCGGTCTGTCCGGC	GTG	2533	1038
5	-4.668	1.628	6	-6.413	GACCTTCGGTCTGTCCGGCGTG	ATG	2536	1035
6	-5.699	1.142	6	-7.444	GATGTTCAAACTCACCGGCCCG	GTG	2557	1014
7	-3.990	1.948	5	-5.990	CCGGGCCGGGTCGATCACGAAG	ATG	2644	927
8	-5.026	1.459	10	-5.720	CAAGATCACCATCGAGAACATC	ATG	2764	807
9	-5.472	1.249	13	-6.518	CACCATCGAGAACATCATGACG	ATG	2770	801
10	-4.343	1.781	13	-5.389	GCTGTACAACGAACAGTCCAAC	ATG	2803	768
11	-5.724	1.130	8	-6.946	CGAACAGTCCAACATGTCGTTC	ATG	2812	759
12	-5.145	1.403	8	-6.366	GCTCAACTTCGTCCTGAACCCG	ATG	2836	735
13	-5.472	1.249	10	-6.167	CCCGTGGTCGGACGACGCGACG	TTG	2863	708
14	-5.997	1.001	11	-6.754	GGACGACGCGACGTTGAACCTC	GTG	2872	699
15	-5.061	1.442	8	-6.283	GAACCTCGTGAAGTCGAACGGC	GTG	2887	684
16	-4.663	1.630	10	-5.358	TTACATTCTCCTGGGCCGGATC	TTG	2953	618
17	-3.990	1.948	11	-4.747	CGACGTCGTCAAGAAGGCTGGG	TTG	3019	552
18	-4.502	1.706	15	-6.104	CCCGGCAGTAACCGACCAGACC	GTG	3160	411
19	-4.502	1.706	18	-6.803	GGCAGTAACCGACCAGACCGTG	ATG	3163	408
20	-4.154	1.871	14	-5.501	GACCGTGATGAACCCCGAGATC	ATG	3178	393
21	-5.924	1.036	14	-7.271	CGGCACACTGATCACCCCGGAG	ATG	3268	303
22	-3.905	1.988	12	-4.741	CGGTCTCAACGGGTACGGGTGC	GTG	3418	153
23	-3.413	2.220	18	-5.714	GTCCGGGCGCAACTCGTTGCG	ATG	3460	111
24	-5.808	1.090	6	-7.553	GGAGCTGCTATACCCCGGCTCG	ATG	3535	36

Figure 4.11

The information displayed here will depend on the setting you choose in the upper right corner of this window. (SD scoring Matrix & Spacing Weight Matrix.) For this display, Kibler 6 and Karlin Medium settings were chosen. Each row in this window represents all of the possible start codons **in the ORF you clicked on** in the Frames window, the corresponding upstream nucleotide sequence, the gene length resulting from that start, and three columns of scores: a Raw SD score, a Genomic Z value, and Final score. Also included is the spacer distance between the chosen SD site and the start. One line's text may be red, and this is a direct result of where you clicked in that ORF. You will find more information about choosing starts in Section 8.

When evaluating your gene calls and choosing between possible start sites, you may find it helpful to have all three windows open at once, as shown in **Figure 4.12** for the Sheen genome.

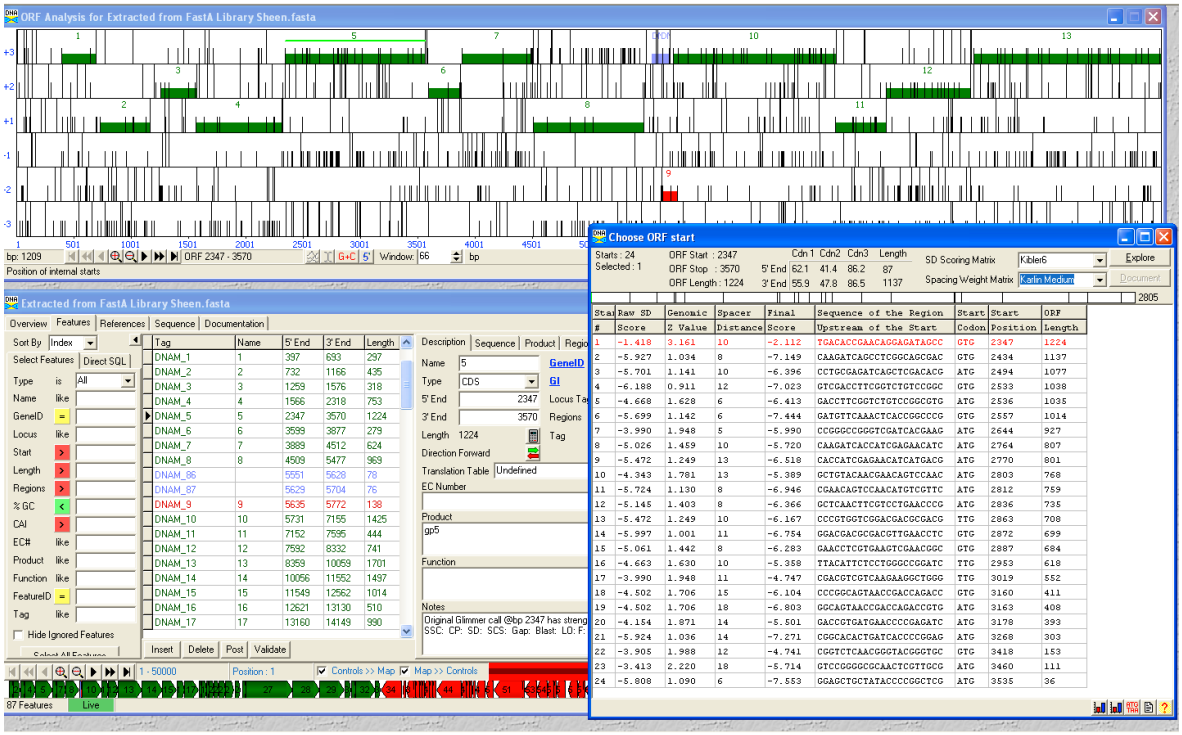


Figure 4.12

4.5 *Running the BLAST function*

When determining the settings for the automated annotation above, we cautioned about the time it takes to run the BLAST function and you may have elected to skip BLASTing. Sooner or later, however, you will need to do this. When you can allow an hour or so for **continuous** Internet connectivity, you should run the BLAST function. To do so, go to:

Genome → BLAST All Genes

- In the dialog box, we recommend that you use the settings shown in **Figure 4.13**.
- You may want to modify these setting and run more than one BLAST. For example, you may want to run a BLAST excluding Mycobacteriophage and Mycobacterium phage. You will have to save each of these in separate .dnam5 files.

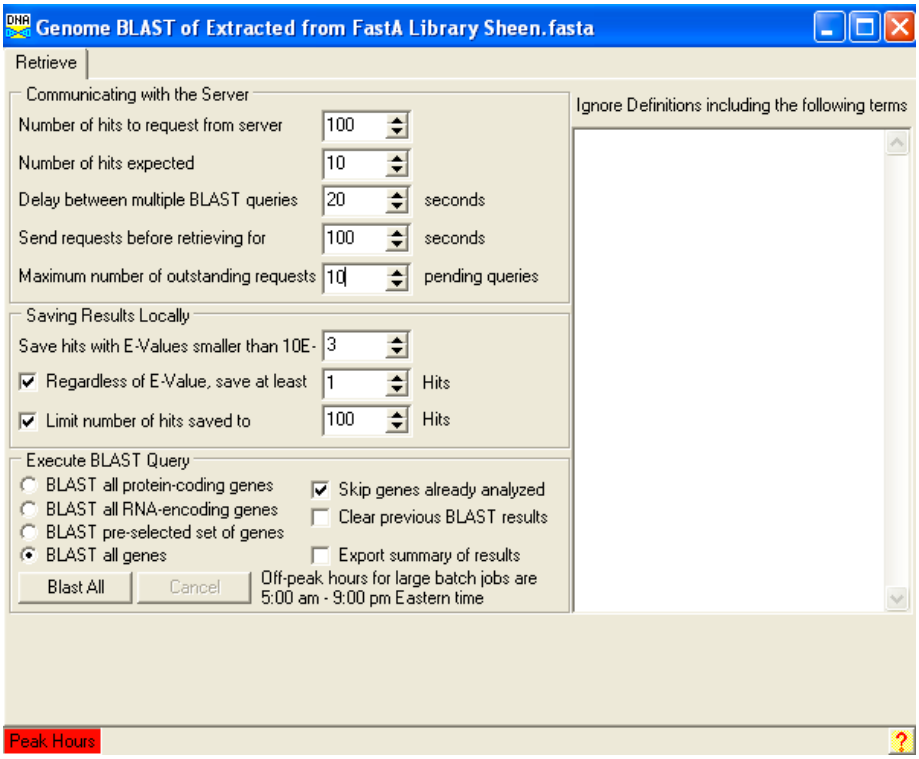


Figure 4.13

- Click on '**Blast All**'.
- The setting are different for Peak and off-Peak hours. This is due to additional parameters put in place by NCBI to accommodate the load put on the NCBI servers as people around the world use BLAST.
- DNA Master will send the predicted protein sequences in your file in batches to the NCBI server, then retrieve the results and store them. Be patient during this process! Windows may briefly indicate that DNA Master is "Not Responding" during this period, but that's because it's processing! You won't be able to use DNA Master until the BLAST is complete. The 'hang time" is most likely a reflection of the extensive retrieval attempts as too many outstanding requests are still pending.

Even though you still only have a draft annotation that was generated automatically, it is very helpful to do the BLAST search **before** finalizing gene calls, because the data will be extremely helpful during the process of annotation refinement. However, as you finalize your file for the Quality Control process you must re-BLAST the genome to ensure that the BLAST data saved in the file is a result of the ORFs of any changes you have made.

When all BLAST searches are complete, DNA Master will report "**Genome BLAST has been completed**" as shown in **Figure 4.14**.

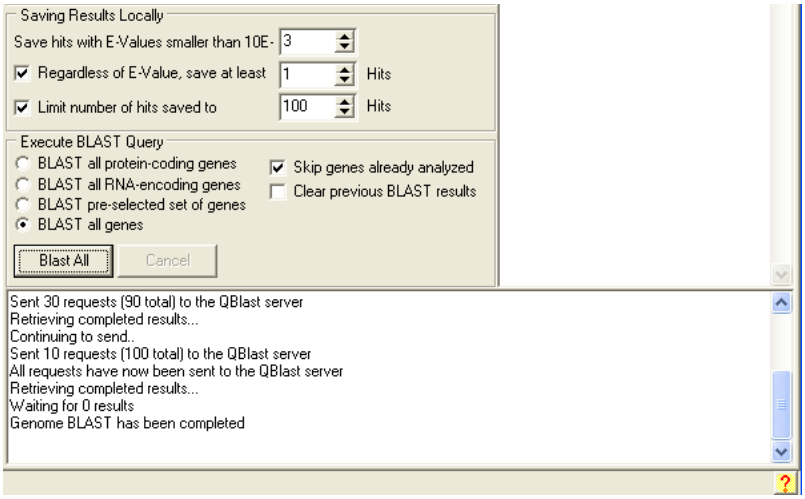


Figure 4.14

- You may now close this BLAST window.
- You can now view BLAST results for any gene by returning to the **[Feature]** tab and selecting a gene, then clicking on the **[[Blast]]** sub-tab to the right. (**Figure 4.15**)
- Save the file with a new **Name**. (This will not auto-save.)

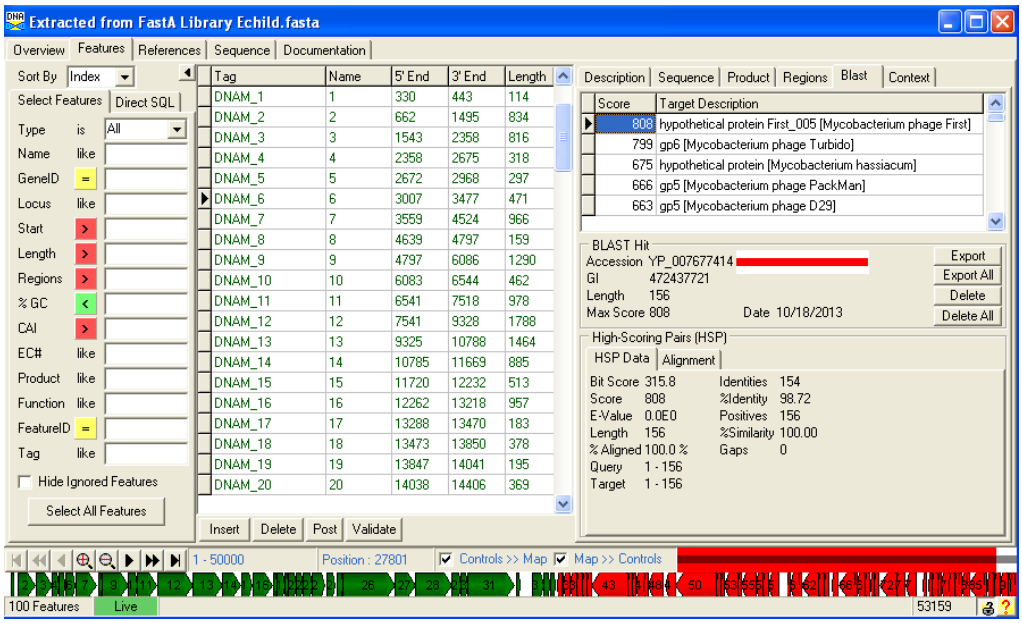


Figure 4.15

In the example above we clicked on gene 6. Under the **[[Blast]]** sub-tab, you can see a window with the BLAST hits listed, with a score and a description. Below that is a pictorial report on the extent of the match (shown as a red bar depicting the part of the gene product – i.e. gp6 in this case – that matches the selected subject). Below that are the data for the hit (HSP Data), and if you click on the **[[[Alignment]]]** sub-sub-tab it will show the actual alignment.

In the example shown in **Figure 4.16**, we clicked on a BLAST hit further down on the list of matches, and then clicked on the **[[[Alignment]]]** sub-sub-tab. Note that you can now see the amino-acid matches in the bottom right pane.

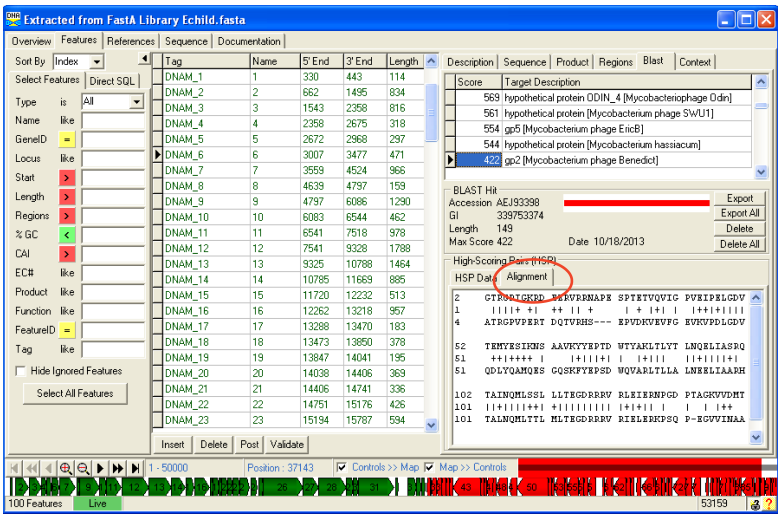


Figure 4.16

- Save your file as described in **Section 4.3** to ensure your BLAST data are stored.

4.6 Re-opening an archived (saved) file

When you save files, Opening archived (saved) files is straightforward. Go to:

File → Open → Archived DNA Master file

- Browse to your saved .dnam5 file and select and open it.

5 Gathering additional information for refining your annotation

There are three additional pieces of data that we recommend gathering at this point. The first is a **six-frame translation** of your sequence labeled with your predicted genes. The second is a **provisional genome map**. The third is a **graphical output of the GeneMark-Smeg** analysis. Depending on your genome, you may also need the **tRNA predictions** from the web-based Aragorn and tRNAscan-SE algorithms. The output of these programs will be used in **Section 8**.

5.1 Generating a six-frame translation

With your genome open in DNA Master (we used Etude below), go to:

Genome → Six-frame translation

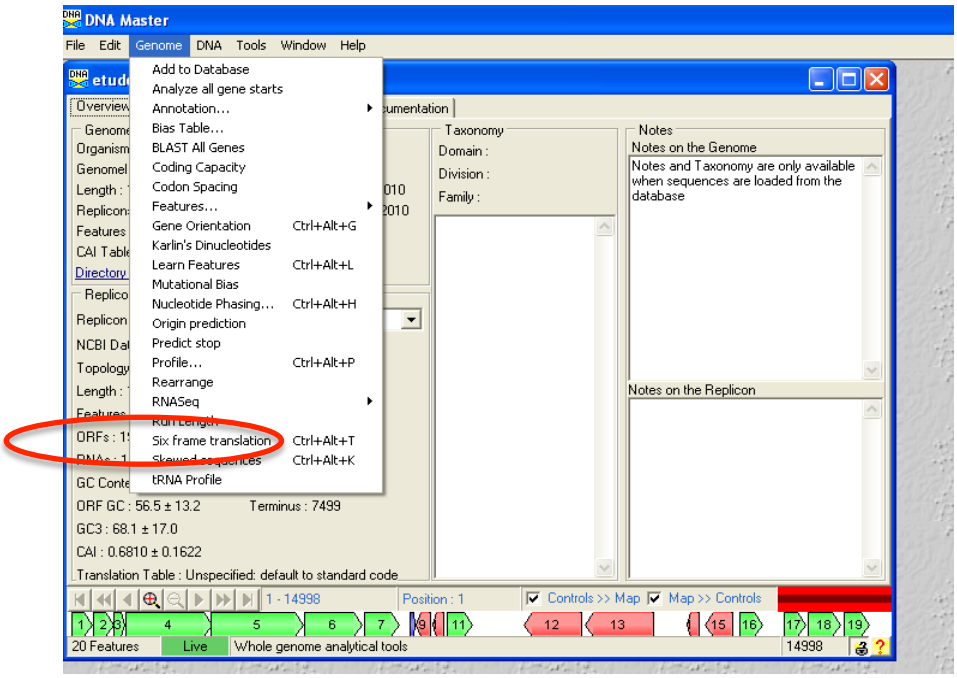


Figure 5.1

The six-frame translation window will open.

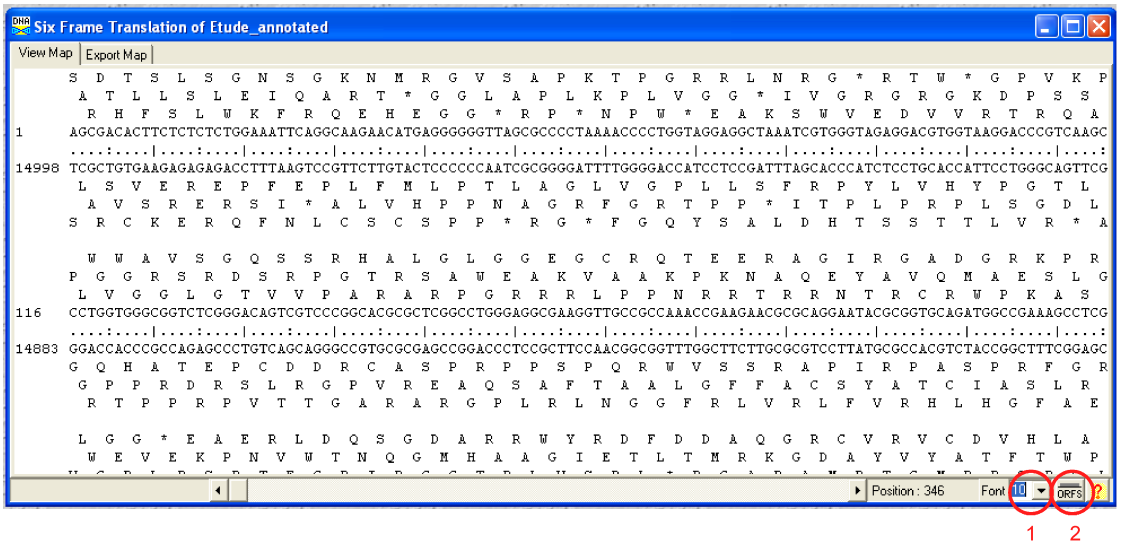


Figure 5.2

- Adjust the size of the font by entering '8' in red circle #1 in **Figure 5.2**.
- Click on the ORFs button in the red circle #2 in **Figure 5.2**.

Note that the ORFs predicted in your auto-annotation are now highlighted. Also note that this window scrolls right and left rather than up and down. When you first click on the ORFs button you may not see highlighted text if there is no gene predicted in the extreme left end of your genome (which is what is shown by default). If you like, you can scroll to the right using the scroll bar at the bottom to see more sequence.

But you can also be assured that your selection has been chosen because the ORFs button at the bottom right is now shown in red (see **Figure 5.3**).

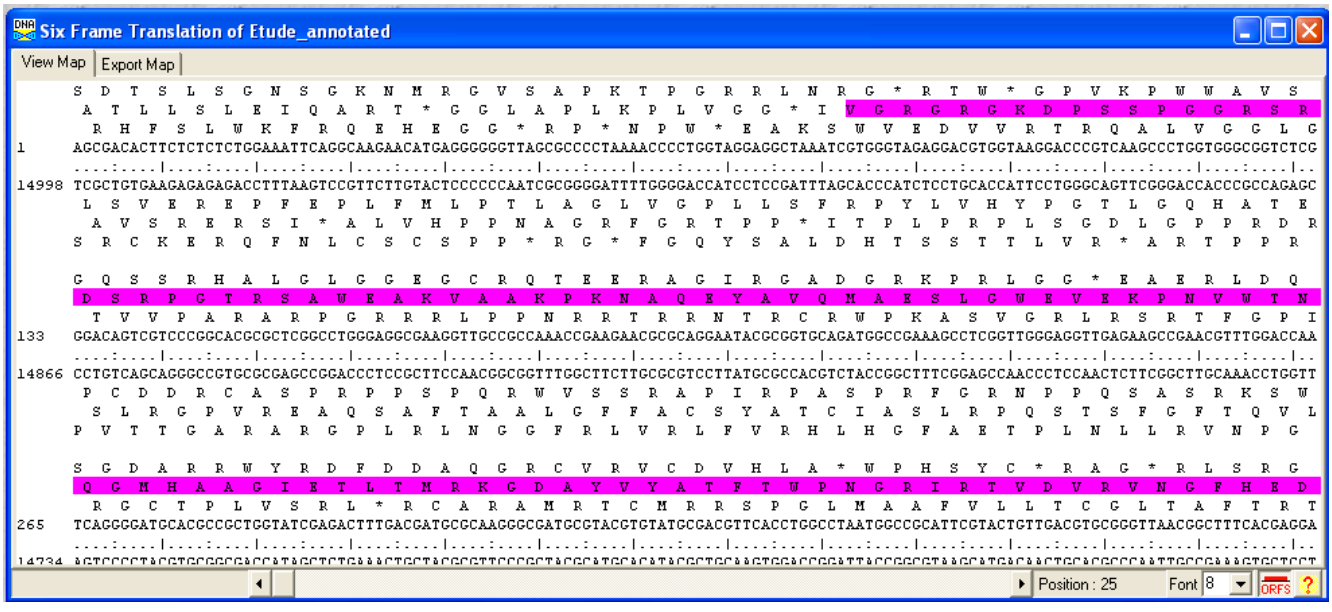


Figure 5.3

Now click on the **[Export Map]** tab at the top left of this window. We recommend using the default settings as shown in **Figure 5.4** below.

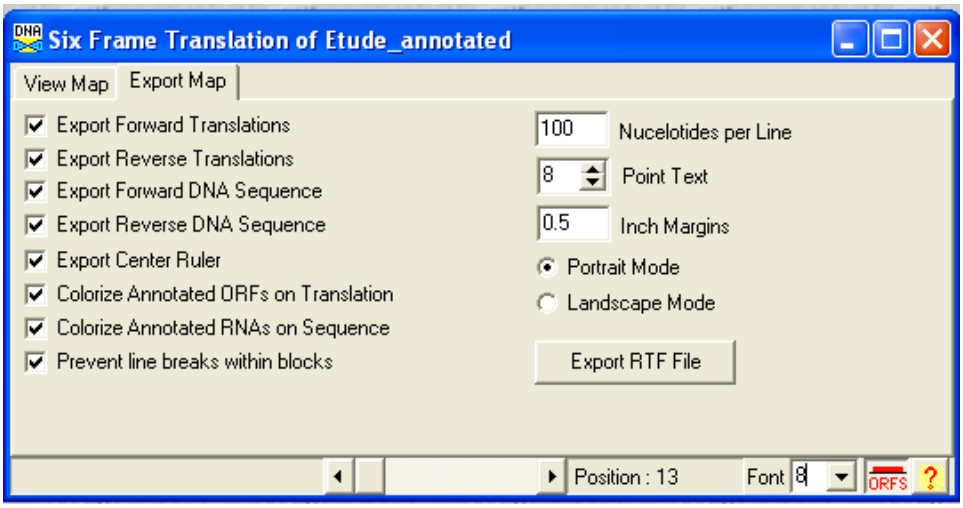


Figure 5.4

- Click the '**Export RTF File**' button.
- In the dialog box that opens, choose a name and location for your translation file, then click '**Save**'.

To view your translation file, it's best to open it with Microsoft Word. Please note that your computer may not be set to open a file with an **.rtf** extension with Word. If not, open the file using the '**Open'** function from within Word. You may want to save as a .pdf in the future to preserve formatting more easily.

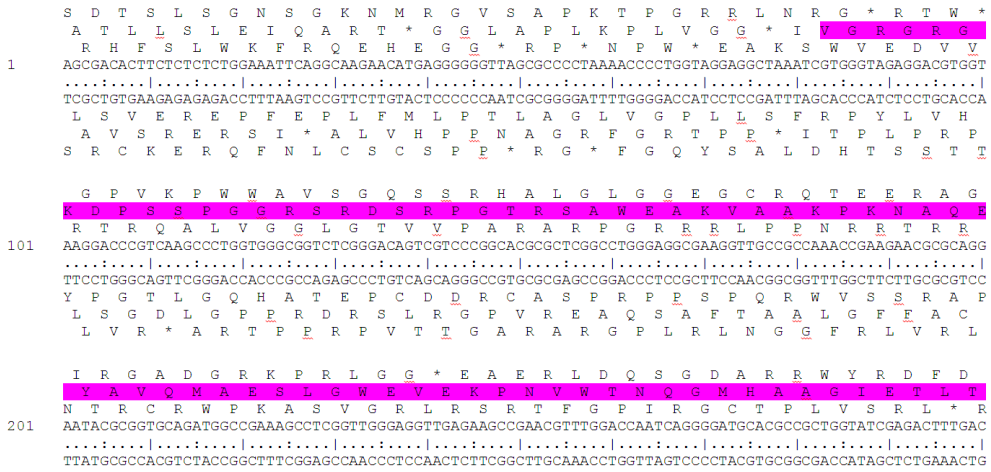


Figure 5.5

The formatting in Word has been set so that you have 100 base pairs per line. Both strands are shown with the coordinates of the bases on the left. There are vertical markers between the strands positioned every ten bases. All six possible translations are shown, with the predicted genes from your automated annotation highlighted.

Note: Because this document can be > 100 pages, we recommend you only print one or two copies to share among a class, or a single copy for an individual. Hard copies are particularly useful when annotating potential ribosomal frameshifts (**Section 8.4.3**).

5.2 *Generating a provisional genome map in DNA Master*

Another useful tool in DNA Master is the ability to make a genome map. This map is not comparative (though you will make a comparative map using Phamerator in the next section), but rather just a separate file of the map shown at the bottom of the sequence panel. Still, it is a useful way to see your gene calls in the context of the entire genome.

To make a genome map (we use mycobacteriophage Timshel below), go to:

DNA → Export Map

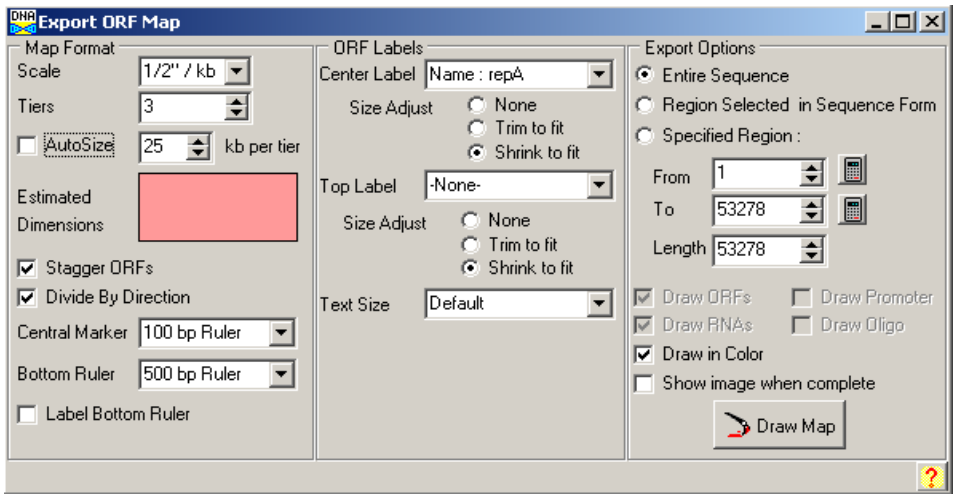


Figure 5.6

- In the dialog box that appears, many options are available. We recommend you use the settings shown in **Figure 5.6**, except that the '**Tiers**' field may need to be adjusted. Three or four tiers are acceptable for a genome of up to about 60 - 80 kb in length. If your genome is larger, increase the number of tiers accordingly.
- Click on '**Draw Map**'.
- Choose a filename and location to save to, then click '**Save**'.

The file will be saved as YourFileName.wmf (Windows metafile). This file can be opened by Preview (on a Mac), Paint, Canvas, or similar drawing programs. Depending on the program, you can manipulate this file in numerous ways. At the very least, you should see an illustration of your genome, similar to one shown in **Figure 5.7**.

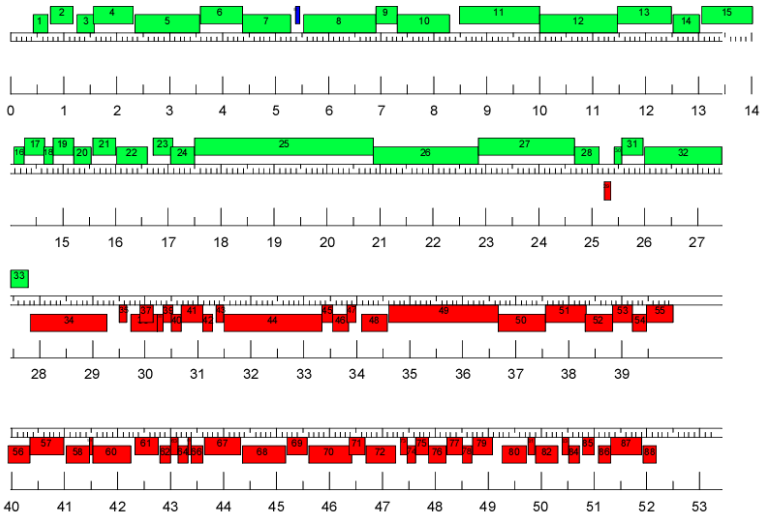


Figure 5.7

5.3 Generating a graph of coding potential using GeneMark

As we noted above, GeneMark is a gene prediction program, and the version embedded in DNA Master runs heuristically, using parts of the genome you enter to train the program to identify coding potential. When using the version on the web, you can:

1. Use an existing coding model to predict the genes.
2. Generate a graph of the coding potential.

The host profile we recommend using is that of *Mycobacterium smegmatis* mc²155, assuming that you used this host to isolate your phage. If you used a different host, you will obviously need to select a different bacterial profile for GeneMark. Even if you isolated a phage using *M. smegmatis* mc²155 as your host, you may find different mycobacterial models (or even Actinomycetales models) yield higher coding potential outputs. As a learning opportunity, you can even use the programs at GeneMark's home page http://exon.gatech.edu/GeneMark/ to obtain a graph of coding potential of the heuristic predictions (like what is imported into DNA Master). Use the version found here (http://exon.gatech.edu/GeneMark/genemarks.cgi).

To run host trained, web-based model GeneMark (we use Mycobacteriophage Sheen below), go to:

- http://exon.gatech.edu/GeneMark/genemarks.cgi . Also found on the Links page of http://phagesdb.org as GeneMark (version 2.5).
- Once on the site, Select '**Browse**', then find and select your sequence file. This is the same YourPhage.fasta file that you imported into DNA Master.
- From the '**Select Species**' dropdown box, select '*Mycobacterium_smegmatis* mc²155' (assuming you are annotating a mycobacteriophage genome).
- In the **'Output Format'** section, check **'LST'** under 'Output format for gene prediction and 'PDF' under output options**.** You can ignore the optional email option (See **Figure 5.8**).
- Click on the '**Start GeneMark**' button at the middle left (See **Figure 5.8**).

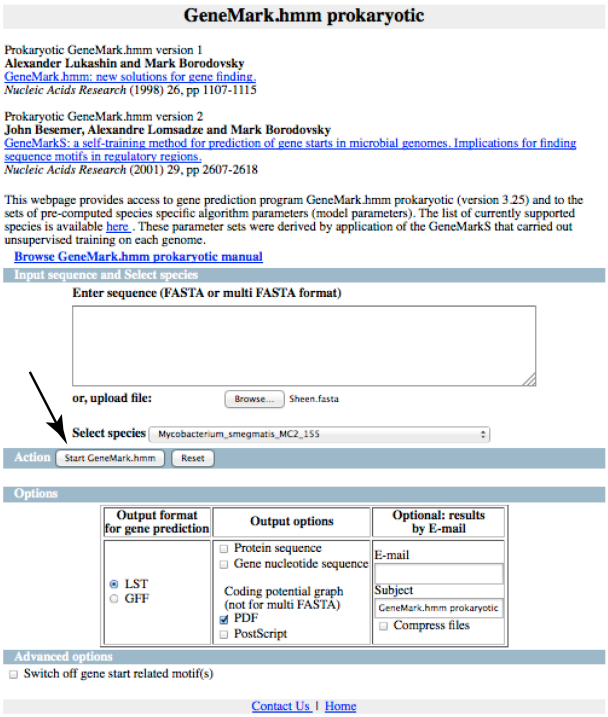


Figure 5.8

Once GeneMark has run, a new window will appear as shown in **Figure 5.9**.

- Click on the link '**gmhmmp.out.pdf**' just below.

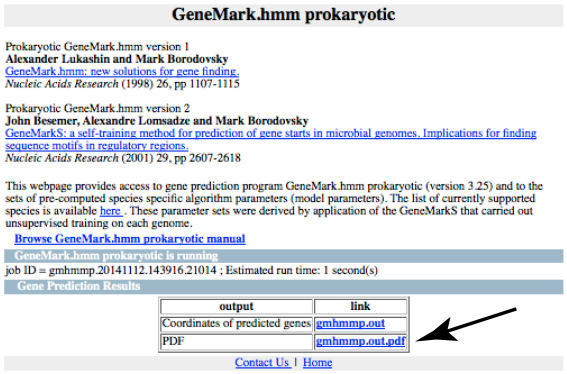


Figure 5.9

- Save and open the .pdf file. We recommend changing the file name to something more useful, like Sheen_smeg.pdf.
- The coversheet of the file provides you with the specifications of that particular run of the program. You will want to check that the specifications match your expectations (See **Figure 5.10**), i.e. check that the following items are identified: the correct sequence (and its length) and that *M. smegmatis* is listed as the Matrix (or whatever host that you picked.

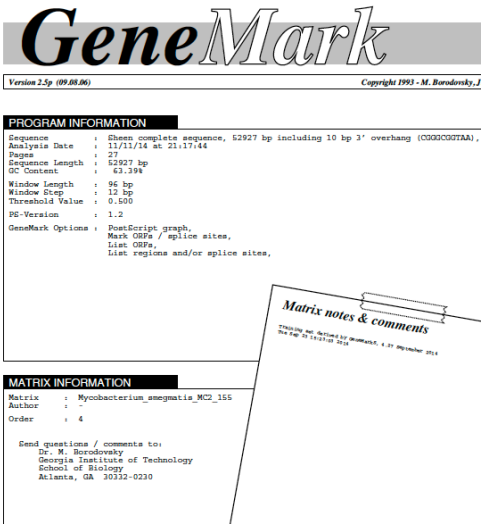


Figure 5.10

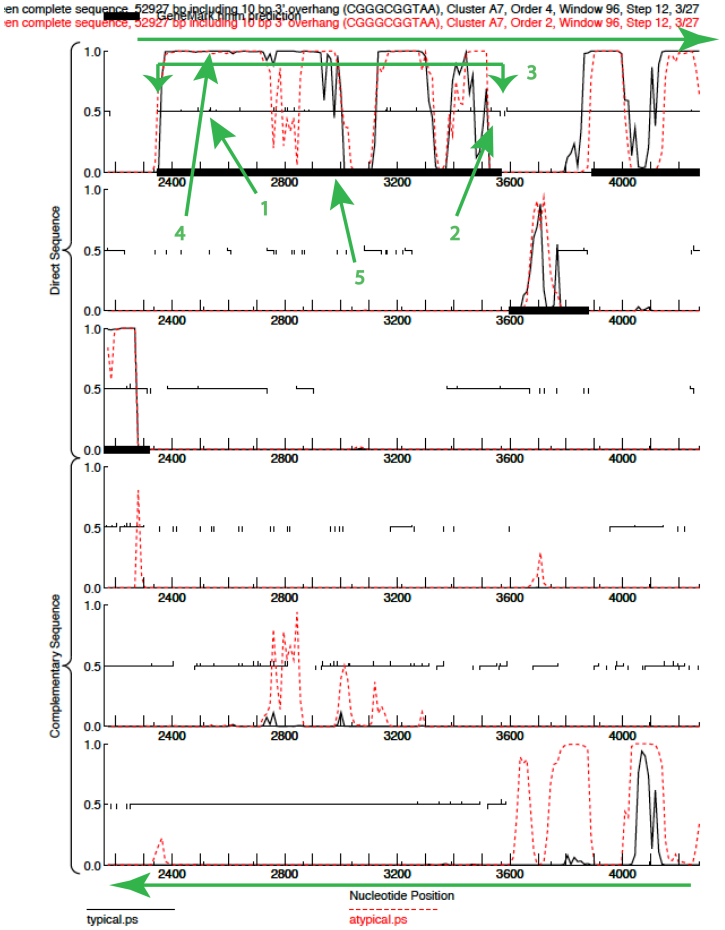


Figure 5.11

We recommend that you **print** this file because it is a good place to make notes as you refine your annotation. Several features of this output are described. (See **Figure 5.11**).

- All six frames are represented and are separated from one another by solid horizontal lines.
- The top three frames are in the forward orientation; the bottom three in the reverse orientation.
- In each frame, the start codons are shown as small upward facing ticks (#1 in figure). Note ATG start upticks are a bit longer than GTG starts while TTG are not shown.
- In each frame, the stop codons are shown as small downward facing ticks (#2).
- The horizontal lines in the middle of each row represent open reading frames (ORFs) (#3).
- A graphical representation of coding potential is shown (#4). Note that the Black lines are typical coding potential and the red lines are atypical.
- The black bars at the bottom of each ORF with coding potential (#5) signify regions that GeneMark predicts as likely coding regions, based on coding potential and positioning of stop codons, but for the most part is of limited utility in gene identification.

6 Phamerator & other Tools to assist with annotation

This section describes the basic applications of two valuable tools in our annotation suite. Phamerator was designed and implemented by Dr. Steve Cresawn at James Madison University. A new method for pham building was developed and implemented by Charlie Bowman at the University of Pittsburgh in 2014.

At present Section 6 contains information about using Phamerator (Section 6.1) and Starterator (Section 6.2) for genome annotation.

6.1 Phamerator

6.1.1 Overview

Phamerator is a Linux-based program that compares phage genomes, their genes, and their gene products, and then displays the results of these comparisons in a variety of useful ways. Phamerator is comprised of two basic parts: an underlying database that contains the results of the comparisons, and a graphical interface to that database.

One of Phamerator's key features is that it groups gene products into "**Phamilies"** (generally referred to as "**Phams**") when the pairwise alignment scores (using BLASTP and ClustalW) are above a defined threshold.

In previous years, we created phamilies (phams) by performing pairwise amino acid sequence alignments between every pair of genes in the database using CLUSTAL and BLASTP. Proteins were assembled into phams if the pairwise alignment scores were above an empirically determined threshold value for each program. This was very computationally expensive---and therefore very time consuming. Incremental building of the 627-member Mycobacteriophage_Draft database required about 6.5e7 pairwise alignments to be performed to build phamilies (or the approximate number of years since the proposed Cretaceous-Paleogene extinction!).

New this Year: In order to continue adding new phages to our database, we switched to a different program, kClust, to build phams. kClust is an alignment-free approach for clustering amino acid sequences that is based on the concept of k-mer profiles. A k-mer profile is built by dividing a protein sequence into small units (called k-mers; ours are about 4-6 aa long) and then creating a list of all k-mers found in the sequence, followed by determining the number of times each k-mer appears in the sequence. This is done for every protein sequence in the database. Then, instead of comparing protein sequences directly, these k-mer profiles are mathematically compared and scored.

The specific pipeline we use produces groupings very similar to the original groupings produced by BLAST and CLUSTALW, with low incidences of both false-positive and false-negative groupings. We accomplish this by using parameters and a workflow that we have optimized through testing and validation, and is loosely based on the kClust_iter pipeline(CITE).

Our pipeline works as follows (**See Figure 6.1**):
First, protein sequence k-mer profiles are compared, and then proteins are grouped based on 75% predicted amino acid conservation with a 25%size cutoff. The size cutoff refers to the minimum size a gene must be to a larger gene to be compared. This grouping collates genes that are very similar, which cuts down on false groupings that were influenced by size differences.

Second, a multiple sequence alignment (MSA) is generated for each group from every sequence in the group and a consensus sequence is determined. Finally, the consensus sequences from the groups, as well as orphams, are then reclustered with kClust solely based on an e-value of 1e-4 with a 50% size cutoff; resulting in a pham profile in Mycobacteriophage_Draft very similar to the one built using the BLASTP/CLUSTALW method; with fewer falsely inflated phams.

Phams are thus groups of proteins with a high degree of similarity to one another.

Phamerator is especially useful for generating and comparing genome maps of multiple phages through the visual interface that displays whole genome nucleotide and protein sequence relationships, as well as the conserved domains within genes.

See the Phamerator Help Menu for the User Manual.

For more on Phamerator and its mechanics, see the following paper.

> Cresawn SG, Bogel M, Day N, Jacobs-Sera D, Hendrix RW, Hatfull GF. "Phamerator: a bioinformatic tool for comparative bacteriophage genomics." *BMC Bioinformatics*. 2011 Oct 12; 12(1):395.
>
> Hauser M, Mayer CE, and Soding J. "kClust: fast and sensitive clustering of large protein sequence databases." *BMC Bioinformatics*. 2013 Aug 15; 14:248

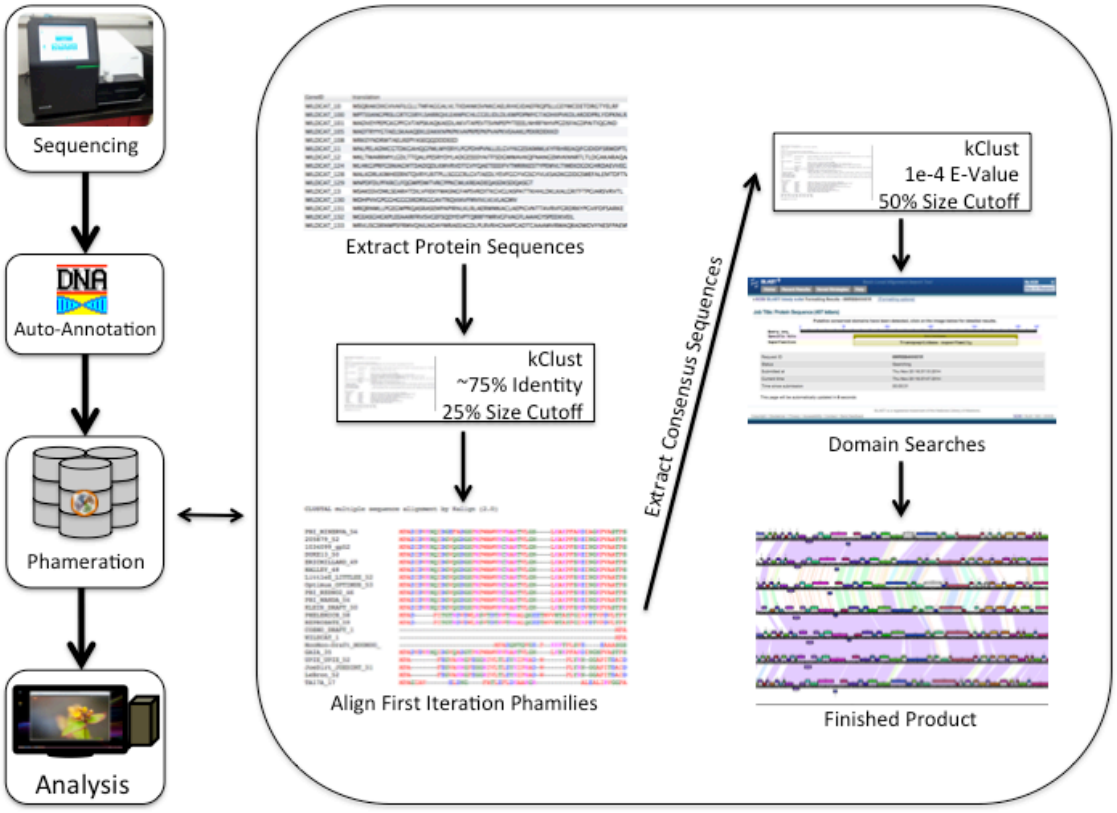


Figure 6.1

Figure 6.1: Figure New Phamerator workflow. After sequencing and autoannotation, genomes are run through the Phamerator pipeline on our servers. First, all protein sequences are extracted and subjected to a grouping using kClust. Then, Multiple Sequence Alignments are made from the resulting groupings. The consensus sequences from these groupings are

removed and added to a second iteration of kClust along with orphams. The results of the second iteration are stored in the database as phamilies. Domain database searches are performed on all of the genes, and then the data is ready to be viewed in the Phamerator client.

6.1.2 Why Phamerator is useful to you at this stage of your annotation

Phamerator maps provide an easy-to-understand representation of how your genome compares to similar genomes. This is useful during annotation because it draws attention to places where your automated annotation diverges from the finalized annotation of a closely related (and often GenBank-published) genome. It also provides a genome-wide perspective and thus a context for the annotation refinement, functional analysis, and other explorations to follow. It is also the primary source of protein functions as denoted in the **Descriptions.** See **Section 10.3.3** for more details.

6.1.3 How did my genome get into Phamerator already?

In order to expedite your annotation workflow, we have taken each newly sequenced genome, generated an automated annotation (just as you did in **Section 4**), and entered all of these files into a Phamerator database that contains all sequenced mycobacteriophages. The database generated is called 'Mycobacteriophage_Draft' because it contains auto-annotated draft genomes along with finalized and published annotations. The auto-annotated genome names are given the suffix "_Draft," so as to distinguish them from the GenBank-quality files. At a later time, when you've refined your annotation and it is submitted to GenBank, your draft annotation may be replaced in Phamerator with your final annotation.

6.1.4 Making Phamerator maps

- Open the Phamerator program. (Allow up to a minute for the main window to appear, as Phamerator will check for new databases when it boots.)
- Click on '**Phages**' in the left 'Sources' pane.
- The name of the current database will be displayed at the top of the window (top red oval in **Figure 6.2**). Make sure the database is "Mycobacteriophage_Draft". If not, go to **Edit → Preferences** and select Mycobacteriophage_Draft from the Database dropdown menu.

Note: Some of the screen shots in this section may not directly match the current version of Phamerator, but the functionality is the same. Enjoy!

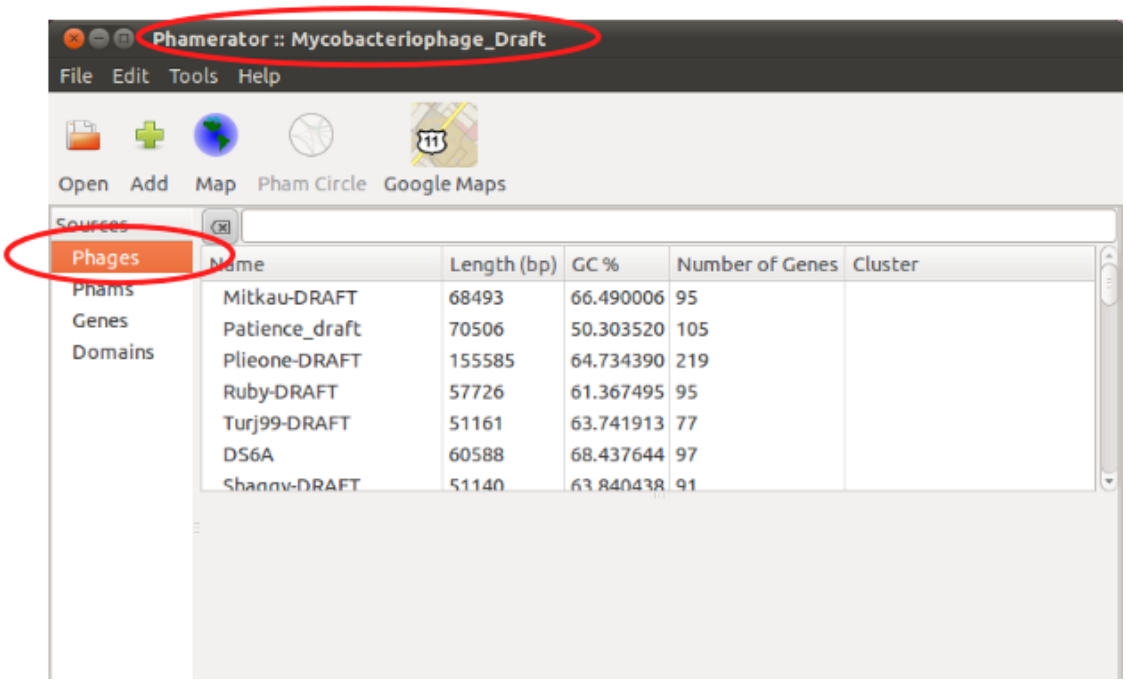


Figure 6.2

You can now choose genomes you want to compare to one another. We recommend:

- Your phage
- Some closely related phages (in the same cluster or subcluster)

You should decide carefully which genomes you want to compare. For example you may not want to compare all of the genomes from a particular cluster if there are a large number. If your phage belongs in a cluster with several different subclusters, you may want to use a representative of each subcluster.

A good rule of thumb is to shoot for no more than about six genomes to start with. You can always return to this and generate more maps as you need them.

- Scroll through the list—or use the search bar—to find your phage.
- Click on it to select it. It will be highlighted.
- To add additional genomes to your selection, scroll through to find the genome you want (if you used the search function, make sure you clear all search terms so that you can see all of the genomes).
- Use Ctrl-click (or equivalent if using an emulator—on Macs it is often Ctrl-Shift-click) to add another genome to your selection. You can also select consecutive genomes in the list by using Shift-click.
- Repeat to select as many genomes as you want to include.
- The phages can also be sorted by simply clicking on the column headers—such as cluster, Length, GC%—to help find relevant genomes.

In **Figure 6.3**, four genomes are currently selected, indicated by the orange highlight.

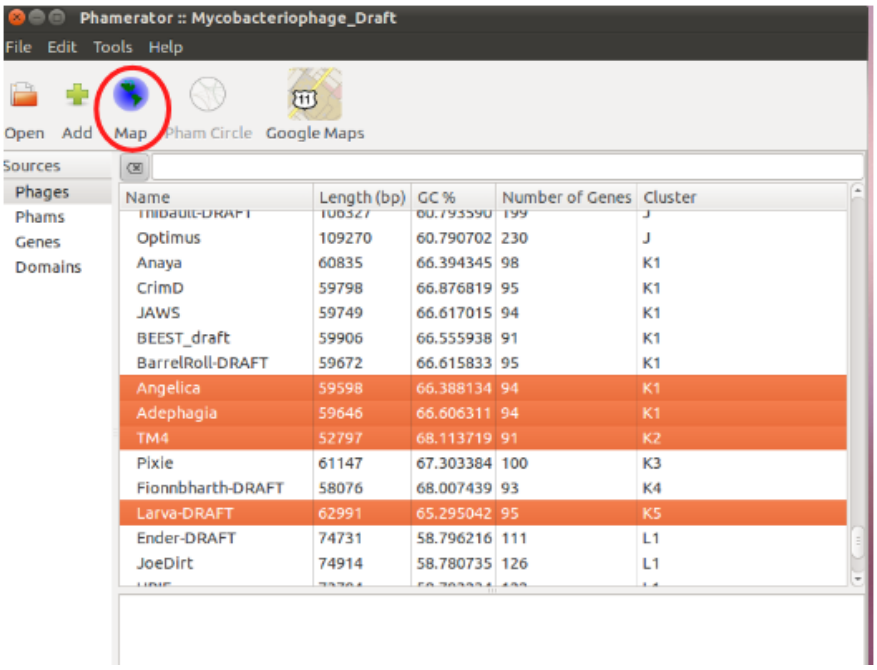


Figure 6.3

- Once you've finished selecting genomes, click on the button that says '**Map**' (red circle in **Figure 6.3**). Be patient, as it can take a minute (or more for a large number of genomes) to generate the map.
- When the map window appears, you will see something like this:

Figure 6.4

Congratulations! You've made a Phamerator map using your phage's draft annotation.

6.1.5 Understanding and using the genome maps made by Phamerator

When the **Genome Map** window appears, you will probably only be able to see a small portion of the genomes. You can resize the window to see more, but you probably won't be able to see the entire picture unless you change the zoom factor. A sample is shown in **Figure 6.5**.

- To see a view of your entire genome, click the '**Zoom Out**' icon at the top left repeatedly until you can see the genome ends.

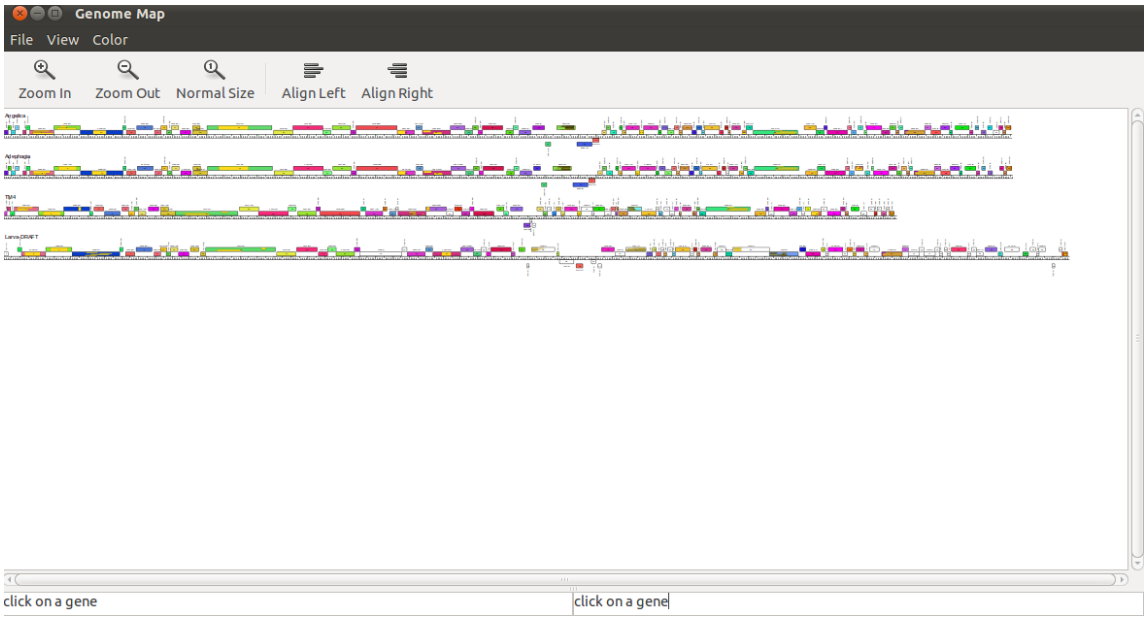


Figure 6.5

Each genome is represented as a hash-marked horizontal bar. Forward-transcribed genes are shown as rectangles above the bar, and reverse-transcribed genes as rectangles below the bar. Each gene is colored according to the **Pham** to which it belongs, making it easy to see relatives in other genomes.

You may have noticed that some genes appear to have smaller yellow boxes within them. These represent matches to the NCBI Conserved Domain Database. These will be particularly useful later when attempting to determine gene functions, but they can be confusing at this stage. Fortunately, Phamerator makes it easy to toggle the display of these domains. Just go to:

View → Show Domains, then click to unselect this option.

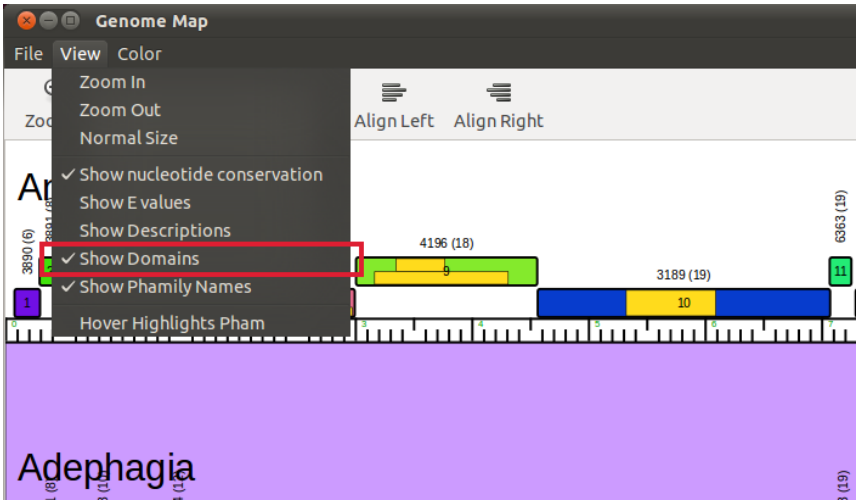


Figure 6.6

Lots of information is displayed on Phamerator maps.

- Click the '**Zoom In**' icon several times to get a closer look.

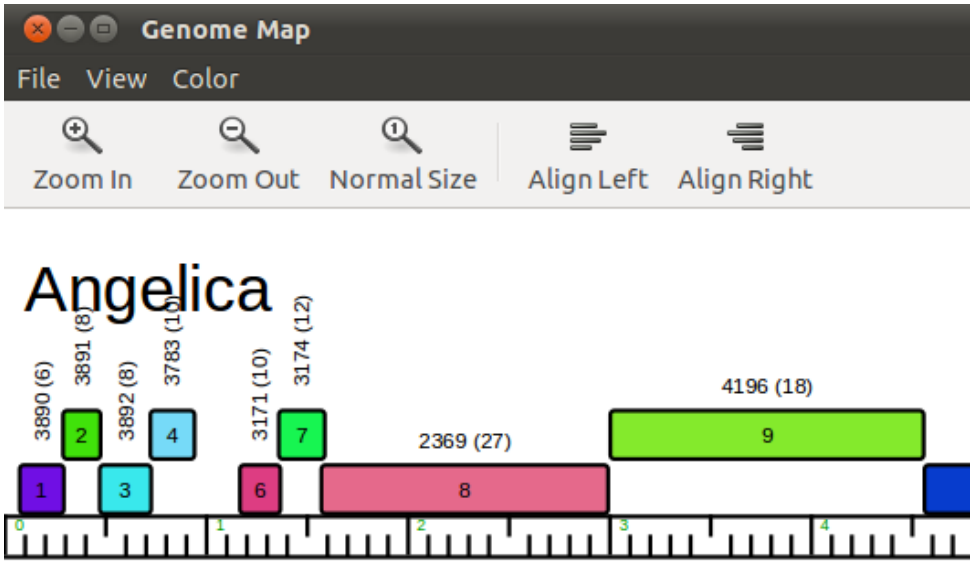


Figure 6.7

Again, the white bar at the bottom represents the genome sequence itself, and is marked with green numbers every 1,000 base pairs (bp). The small hash marks coming up from the bottom show 100 bp intervals, while the ones coming down from the top show 500 bp intervals.

Each gene's box has a number within it that represents that gene's number in this genome. There are also two numbers above each gene; the first is the number of the Pham this gene belongs to, and the second—in parentheses—is the total number of members of that Pham.

Putting all this together, we can determine that Angelica's gene 8 begins at ~1600 bp, ends at ~3000 bp, is a member of Pham 2369, and that there are 26 other members in that Pham:

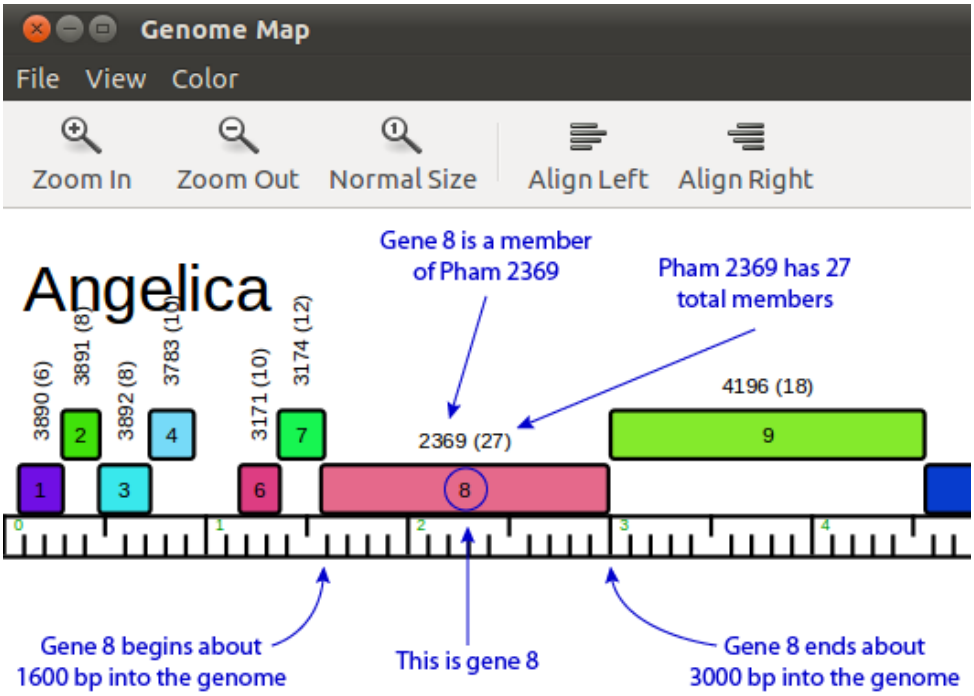


Figure 6.8

6.1.6 Viewing nucleotide sequence similarities in Phamerator

A NOTE ON TWO DIFFERENT TYPES OF SIMILARITY

Nucleotide sequence similarity is a comparison of the **DNA sequence** (A, C, G, T) of two **genomes**. It is often determined by running BLASTN. On Phamerator maps, nucleotide similarity is shown by colored vertical boxes between genomes.

Protein similarity is a comparison of the **amino acid sequence** of two **proteins**. It is often determined by BLASTP or ClustalW. On Phamerator maps, protein similarity is shown by similarly colored gene boxes.

Phamilies, or **Phams**, are determined based on **protein similarity and NOT nucleotide similarity**.

Don't confuse these two types of similarity, or you may misinterpret the data that Phamerator is showing!

While Phamerator was conceived to compare protein sequences to other protein sequences, it can also show nucleotide sequence similarity between genomes. To enable this function:

View → Show nucleotide conservation should be checked (as in **Figure 6.9**).

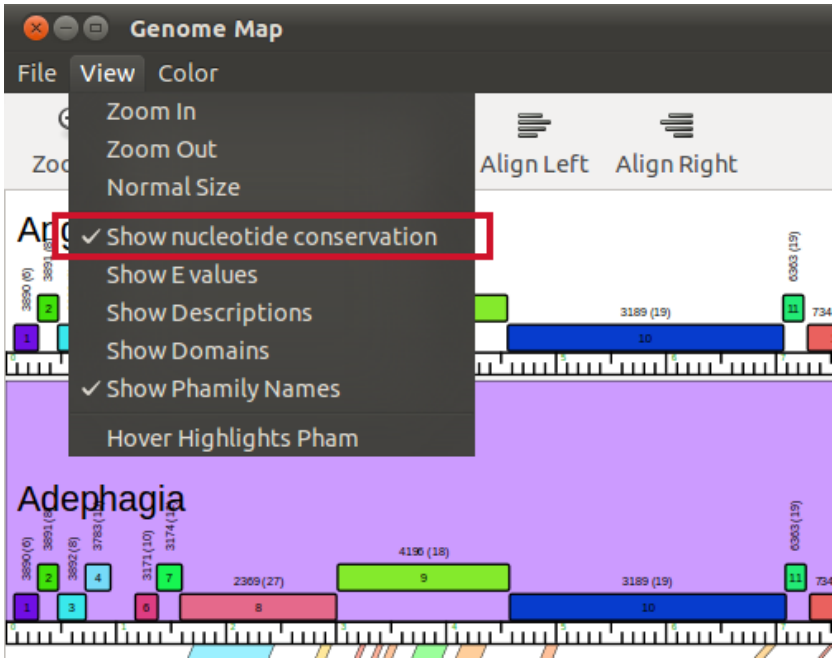


Figure 6.9

Once you've turned on '**Show nucleotide conservation**', you may see colors between the genomes on your map, as shown in **Figure 6.10**.

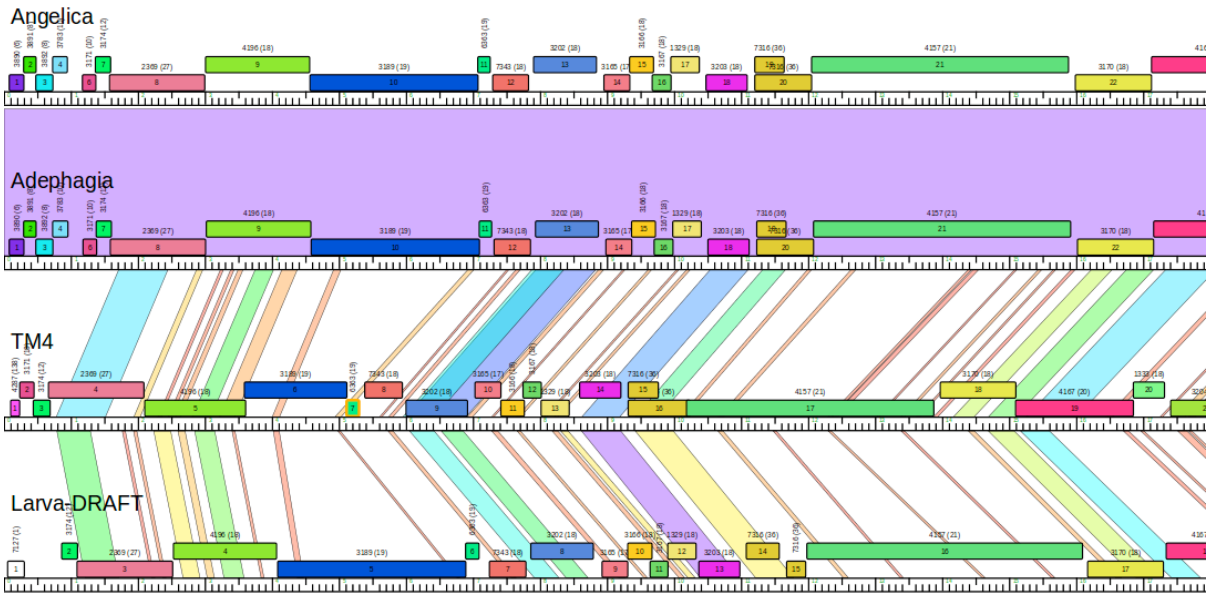


Figure 6.10

Nucleotide sequence similarity is shown by the (often slanted) shaded regions (boxes) **between genomes**. Each box represents one BLASTN alignment, and is colored based on its E value, with violet representing the best matches (lowest E values) and red the worst matches (highest E values). White areas indicate that there is **no** nucleotide similarity in those regions.

Looking at the screenshot above, it is apparent that the top two phages (Adephagia and Angelica) have widespread nucleotide similarity to one another, as indicated by the solid

purple between the two genome maps. The other two phages shown (TM4 and Larva) have multiple regions of nucleotide similarity, though these areas are interrupted by dissimilar (white) areas and have higher E values. This segmented similarity is a reflection of what you saw in the BLAST searches performed earlier. The top two genomes are members of Subcluster K1, while the bottom two are members of other subclusters within Cluster K.

Phamerator-generated maps can be extremely helpful when trying to evaluate a gene start codon in your novel genome that (for example) produces a bigger gene than in the compared genomes. A quick look at the Phamerator-generated map lets you know that the upstream sequence does or does not have sequence similarity.

6.1.7 Other Phamerator features

There are many other functions in Phamerator. Several examples are below.

1. Click on the colored portion of any gene's box to select it, and the nucleotide and amino acid sequences of that gene are shown in the bottom panels.
2. You can move the order of genomes around in the display. This is important, because the nucleotide similarities are only displayed by comparing two adjacent genomes in the display. To do this, click and hold on the **NAME** of a phage you want to move (it is on the extreme left, and you may need to scroll over to it), then drag the genome either up or down to where you want it and release it.
3. You can move a genome to the left or right to better compare it to its neighbors. To do this, Ctrl-Click-hold on the **NAME** of the phage (on a Mac, this might be Ctrl-Shift-Click-hold), then drag to the left or right and release.
4. You can also align genes from multiple genomes, such as those within a particular Pham. For example, you may have noticed that gene 13 in Adephagia is in the same Pham as gene 9 in TM4. Select gene 13 from Adephagia, then Ctrl-click to select gene 9 from TM4, and verify that both genes are highlighted. Then press the "Align Left" or "Align Right" button at the top of the genome map.
5. You may want to also explore the '**Hover Highlights Pham**' function, available in the **View** menu.

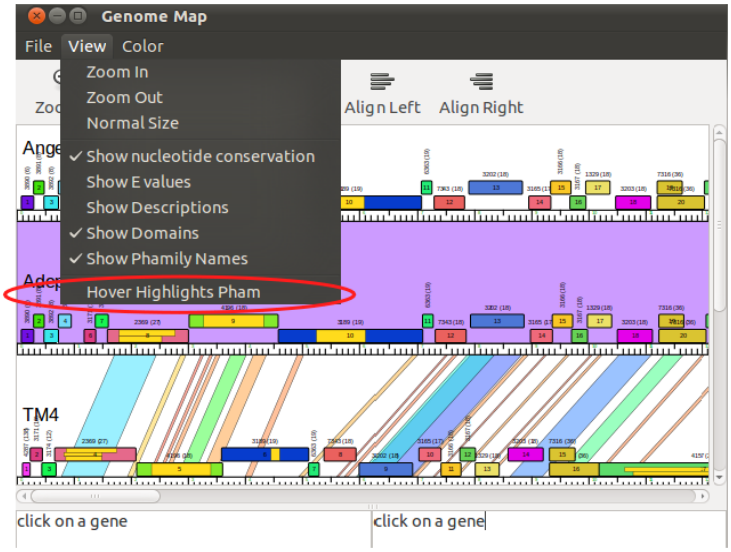


Figure 6.11

This function's use is that when your mouse hovers over any gene, only the gene members of that particular Pham are shown in color, while all others go white. This is a very useful function for easily seeing gene conservation or loss in different genomes.

6.1.8 Saving Phamerator maps

Finally, if you would like to save the map as a file, from the Genome Map window go to:

- **File → Save As**
- Enter a name and select your desired file type (pdf files are a good choice).
- Click '**Save**'.

6.2 Starterator

6.2.1 Overview

A companion program of Phamerator, Starterator uses comparative genomics to highlight conserved start codons for a given pham. Often, the scientific evidence for selecting a specific start codon for a gene is not in agreement (discussed in section 8.4). However, by analyzing that gene as part of a larger multiple sequence alignment, a start codon that is common to all genes in the alignment can sometimes be found.
Starterator aligns the longest possible ORF for each gene (from stop codon to stop codon) in a pham using ClustalW, and produces a graphic that shows all of the possible starts for all of those ORFs in the pham. It is possible to run Starterator on multiple genes or phams at a time, however, the more genes that are selected, the longer the program will take to finish. An entire genome can take several hours. During classroom annotation, it may be more advantageous to run Starterator on each gene individually as the need arises; or to generate a single .pdf of an entire genome outside of class-time to be shared in a future class.

A more detailed guide for the installation and operation of Starterator can be found here at the Software page of PhagesDB and Seaphages.

6.2.2 Interpreting Starterator results:

The Starterator output of a pham is comprised of a graph followed by a text summary.
In the graph below, each horizontal bar, or "track" in the output represents a single ORF---unless some of the phages in the alignment have identical sequences. Identical sequences are then pooled together in the same track. The length of all of the bars is determined by the longest ORF in the pham (in the example below, the ORFs in tracks 1, 6, 14, 16, and 18 are longer than the rest of the ORFs in the pham). The pink regions of the bars are included in the Clustal alignment, the white regions represent gaps in the alignment. The multi-colored vertical bars in each track represent all the possible start codons for each ORF. The starts are numbered from left to right in order of appearance. Starts that are aligned between tracks have the same number and the same color. Starts that are currently annotated in Phamerator are colored blue, the other start colors are selected randomly. The **Figure 6.12** was generated by running Starterator on Sisi gene 5:

Figure 6.12

While the genes in the pham above are very similar to each other—as shown by the perfect alignment of starts from start "6" through the ends of the ORFs--- the region upstream of the start is not. All of the genes in this pham have start "6" in common. However, start "6" has not been selected as the start in the annotation in Phamerator in a number of genes. Many of these genes are the lengthier genes in the pham, and these starts may have been chosen by annotators who selected the longest ORF possible.

Following the graph is a report that lists all the genes that were included in the alignment and the track each is represented by:

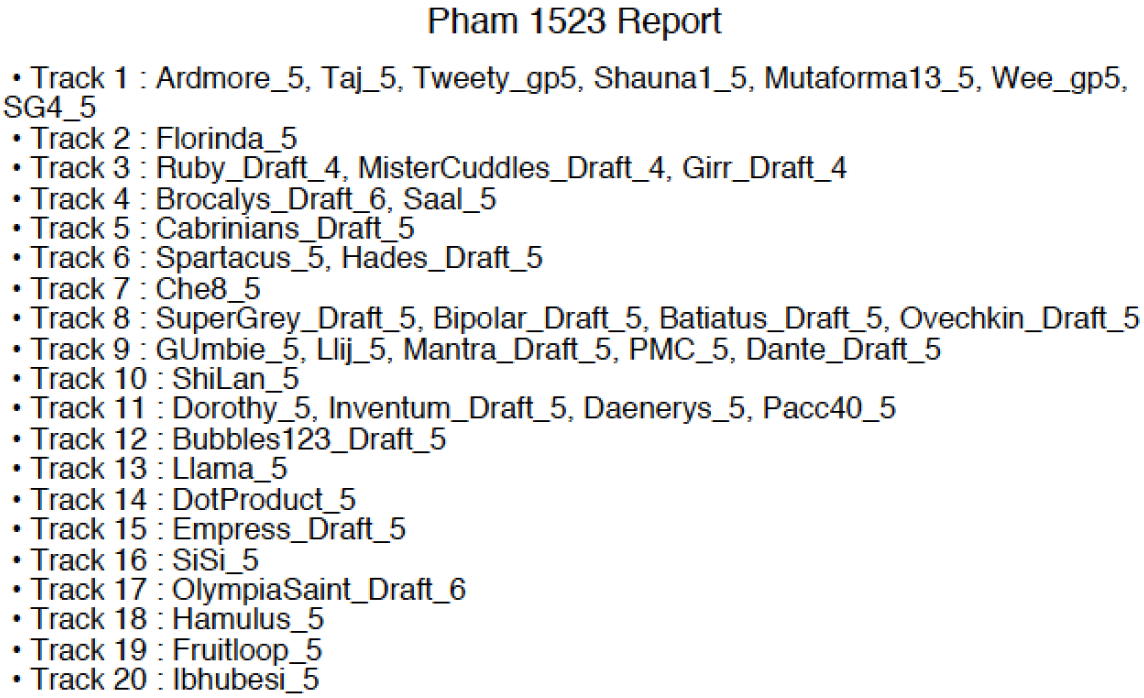


Figure 6.13

Sisi gene 5 is Track 16 of the graph.

The final component of the report lists a "recommended start"; based on the most frequently annotated start(s) found in Phamerator, along with all the possible starts for the Starterated gene(s).

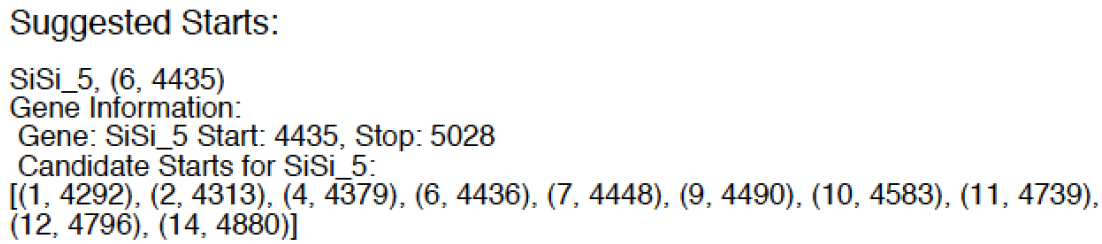


Figure 6.14

Starterator suggests that Sisi gene five should use start 6, at Sisi genome coordinate 4435.

It is important to remember that the Phamerator database contains draft annotations, and therefore a commonly selected start found by Starterator may be an artifact of unrefined auto-annotations generated by the computer gene-calling programs.

7 Guiding Principles of Bacteriophage Genome Annotation

7.1 Overview

Genomes are best annotated when you understand their context. Their context can include how similar or different they are to other phages. What cluster are they a member? How similar are the phages of that cluster? How similar is your phage to the next closest phage? You may want to BLASTN your sequence on PhagesDB, align your sequence with its closest match at BLASTN at NCBI (use the align two sequences tool and format using display with "Pairwise with dots for identities"). You will want to use your Phamerator data for nucleotide and protein comparisons, and the DNA Master Genome Comparison tool (See Protocols -> Further Discovery -> Exploring Bacteriophage Biology). Once you have an overview of your phage genome, you are ready to start calling the genes.

Though the automated annotation you have created using DNA Master will usually identify more than 90% of genes correctly, some genes will need to be manually added, modified, or deleted. Therefore, all gene predictions must be reviewed to identify those that must be changed. In this section, we provide a set of principles that should guide you as you evaluate and improve upon your draft annotation.

It is helpful to think of the process of evaluating your draft annotation's gene calls as an application of these principles: together they will help you make the best possible gene predictions. It is essential to understand that any annotation consists of making a **prediction** as to how the genetic information is organized and used. In the absence of experimental evidence to support a given gene call, there is no right or wrong answer; there are, however, well-supported or ill-supported predictions.

As with any set of principles, the ones presented here will conflict with one another at times. It's your job to weigh one against another and make the best gene calls possible.

Because of the importance of these principles, this section is dedicated wholly to presenting them. Read them carefully before beginning an annotation, and keep them nearby as you work.

7.2 The Guiding Principles

The following two pages list the principles themselves. As mentioned above, we recommend that you print those two pages, read them carefully, and keep them close at hand as your refine your gene calls. Skip ahead to **Figure 8.1**. This is a diagrammatic representation of the work involved in annotating a genome for GenBank submission. The gene prediction analysis described in the Guiding Principles are part of the first two boxes of that diagram (Sections 8.4.1 and 8.4.2).

Because these are principles, and not unbreakable rules, you'll see words like "usually," "generally," and "typically" used quite frequently. Remember that phages are famous for finding exceptions to "rules", so very little is truly set in stone.

GUIDING PRINCIPLES

OF BACTERIOPHAGE GENOME ANNOTATION

1. In any segment of DNA, typically only one frame in one strand is used for a protein-coding gene. That is, each double-stranded segment of DNA is generally part of only one gene.

2. Genes do not often overlap by more than a few bp, although up to about 30 bp is legitimate.

3. The gene density in phage genomes is very high, so genes tend to be tightly packed. Thus, there are typically not large non-coding gaps between genes.

4. Protein-coding genes should have coding potential predicted by Glimmer, GeneMark, or GeneMark Smeg. Start sites are chosen to include all coding potential. These are, by far, the strongest pieces of data for predicting genes.

5. If there are two genes transcribed in opposite directions whose start sites are near one another, there typically has to be space between them for transcription promoters in both directions. This usually requires at least a 50 bp gap.

6. Protein-coding genes are generally at least 120 bp (40 codons) long. There are a small number of exceptions. Genes below about 200 bp require careful examination.

7. Switches in gene orientation (from forward to reverse, or vice versa) are relatively rare. In other words, it is common to find groups of genes transcribed in the same direction.

8. Each protein-coding gene ends with a stop codon (TAG, TGA, or TAA).

9. Each protein-coding gene starts with an initiation codon, ATG, GTG, or TTG. But note that TTG is used rarely (about 7% of all genes). ATG and GTG are used at almost equivalent frequencies.

CONTINUED…

GUIDING PRINCIPLES

10. An important task is choosing between different possible translation initiation (i.e., start) codons. The best choice of start site is gene-specific, and gene function and synteny must be carefully considered. As phage genes are frequently co-transcribed and co-translated, less weight may be given to optimal ribosome binding site sequences in start site selection. Identifying the correct start site is not always easy and is predicated on the following sub-principles:

    a. The relationship to the closest upstream gene is important. Usually, there is neither a large gap nor a large overlap (i.e., more than about 7 bp). If the genes are part of an operon, a 4bp overlap (ATGA), where a start codon overlaps the stop codon of the upstream gene, is preferred by the ribosome. Therefore RBS scores may have little bearing in this type of gene arrangement.

    b. The position of the start site is often conserved among homologues of genes. Therefore, the start site of a gene in your phage is likely to be in the same position as those in related genes in other genomes. But be aware that one or more previously annotated and published genes could be suboptimal, and you may have the opportunity to help change it to a more optimal one. Homologues in more distantly related genomes (those of a different cluster) may prove more informative because alternate incorrect start sites are less likely to be conserved. Use Starterator!

    c. The preferred start site usually has a favorable RBS score within all the potential start codons, but not necessarily the best. A notable exception is the integrase in many genomes, which has a very low RBS score. Our experimental data suggests that some genes do not have an SD sequence.

    d. Manual inspection can be helpful to distinguish between possible start sites. The consensus is as follows: **AAGGAGG – 3-12 bp – start codon**.

    e. Your final start-site selection will likely represent a compromise of these sub-principles.

11. tRNA genes are not called precisely in the program embedded in DNA Master, and require extra attention. (Please refer to **Section 9.5**.)

8 Gene by gene: evaluating and improving your draft annotation

8.1 Overview

This section describes the heart of the matter: how to go through a draft annotation, one gene at a time, and decide whether or not the automated annotation called the gene correctly. You will spend most of your annotation time in this section, because you'll need to follow the steps here between 50 and 250 times per genome, once per gene!

If you've been following this guide step-by-step, you probably have all the items listed below ready to use. If you've jumped directly to this step, you may want to gather the items listed below to assist you as you go.

1. Your draft annotation file (from **Section 4**) open in DNA Master. (It is helpful to have DNA Master's Frames window open as well, with the windows arranged as shown as the last figure in **Section 4.4.4**.)

2. A printout of the Guiding Principles of Bacteriophage Annotation (**Section 7.2**).

3. Phamerator running, preferably with a map displaying your genome and related genomes (**Section 6**).

4. Your GeneMark outputs, most notably the one generated using a host (*M. smegmatis*) as the target. (**Section 5.3**).

5. (Optional) A printout of your DNA Master-generated map (**Section 5.2**).

6. (Optional) A printed six-frame translation of your sequence (**Section 5.1**).

One useful configuration is to have a pair of annotators work together on a genome, using two computers, one with DNA Master running, and the other with Phamerator.

8.2 Button-pushing mechanics reserved for Section 9

The goal of this section is to help you **decide** what modifications need to be made to your draft annotation. In order to keep this section manageable and streamlined, we've moved the detailed **mechanics** (button-pushing) of many of these operations to **Section 9** of this guide.

Section 9 should be used more as an à-la-carte reference than as a step-by-step guide. For example, you probably won't need to read **Section 9.4.1** about properly annotating a programmed translational frameshift until you come across one during your annotation review.

8.3 Decision Tree for evaluating the draft annotation

To help clarify how to use Sections 8 through 12 of this guide, a decision tree is shown in **Figure 8.1**. There are three beginning tracks depending on what feature of your genome you're currently investigating: one for **Protein-Coding Genes** (**Section 8.4**), one for **Gaps in the Annotation** (**Section 8.5**), and one for **Special Considerations** (**Section 9.4**).

Blue boxes are **decision** points, most of which are covered in the rest of **Section 8**. To answer the question in each decision box you'll need to keep in mind the Guiding Principles described in **Section 7** of this guide as well as the rest of the information in this section.

Purple boxes are **action** points where you implement the changes you've decided on. These actions are described in detail in parts of **Section 9**.

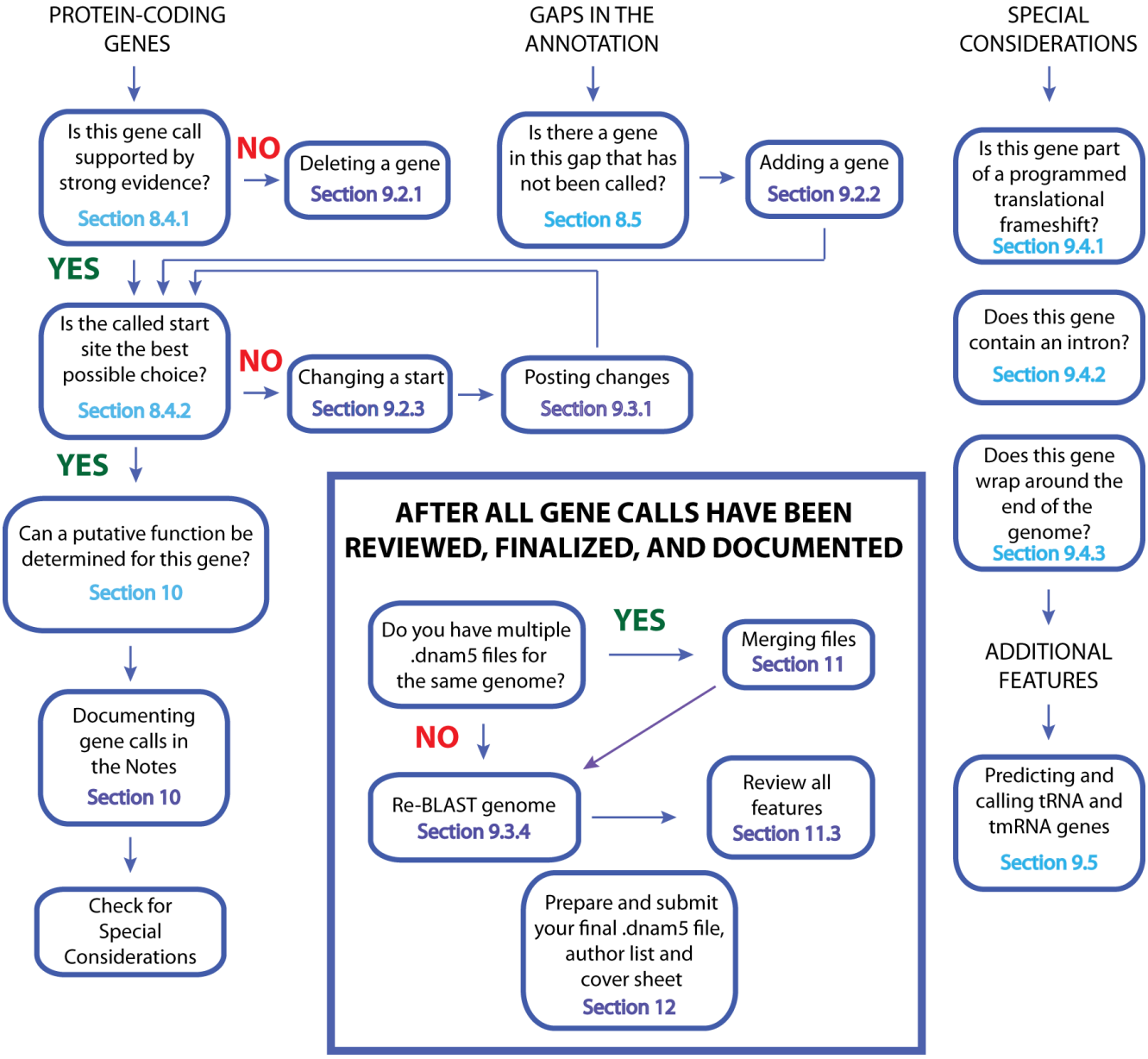


Figure 8.1

8.4 *Evaluating protein-coding gene calls*

The vast majority of features you will need to investigate are protein-coding genes, so you will use this section extensively. The first few genes you review will probably take some time as you become quite familiar with the process, but as you gain experience things will move faster.

It is best to start with your second open reading frame, which will typically be called gene '2' in the DNA Master feature table. We recommend skipping gene 1 until you have some practice. With no upstream sequence, some rules do not apply. Just remember to revisit this gene later!

In evaluating the veracity of the prediction of this gene that was performed automatically by Glimmer and GeneMark, there are several questions you should ask, described in the following sub-sections. We'll use a sample gene, but you can proceed with your genome from here on.

It is also recommended that—in accordance with good lab practice—you keep notes of your thoughts and decisions as you proceed. You'll use them to enter your final Notes (**Section 9.6**).

8.4.1 Is the designation of this ORF as a gene well-supported?

If it's not already selected, click on the **[Features]** tab.

In the central column, click on the gene in question to select it. A small black triangle will appear to the left of that gene, indicating that it is active.

Look at the "Notes" field under the **[[Description]]** sub-tab.

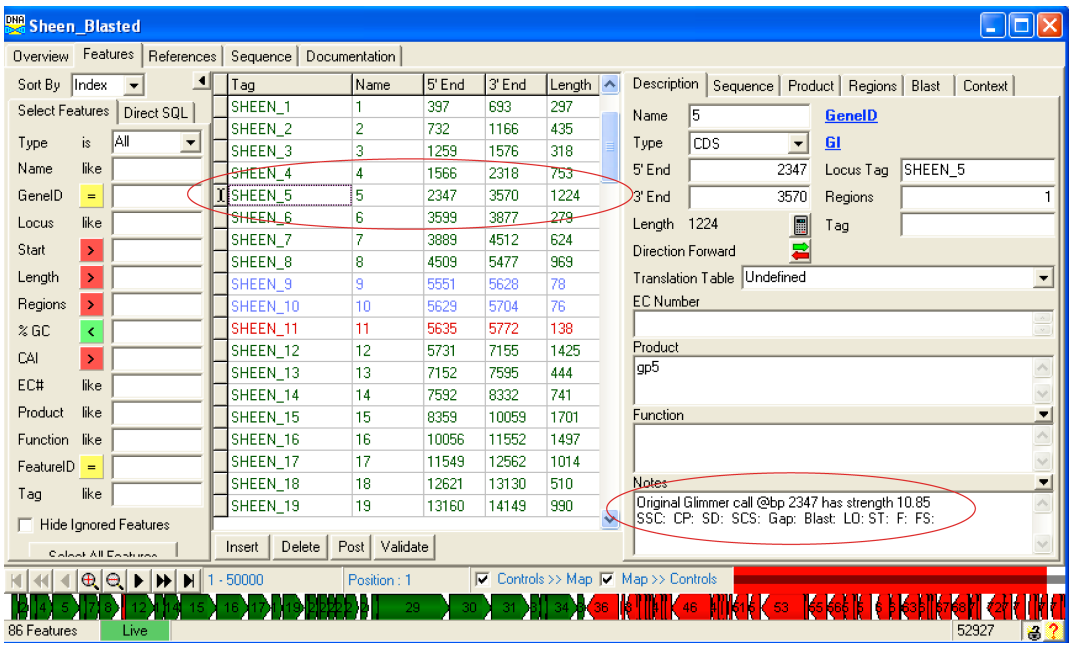


Figure 8.2

The notes should report whether Glimmer and/or GeneMark made the prediction. In the example above, both Glimmer and GeneMark did predict the gene with the same start. (Remember that if both programs agree, only one program's output is reported.) The gene was called by both programs, which supports its legitimacy. Good so far.

Find this gene in your GeneMark-Smeg output, and check if there is coding potential that supports this gene call (Figure 8.3).

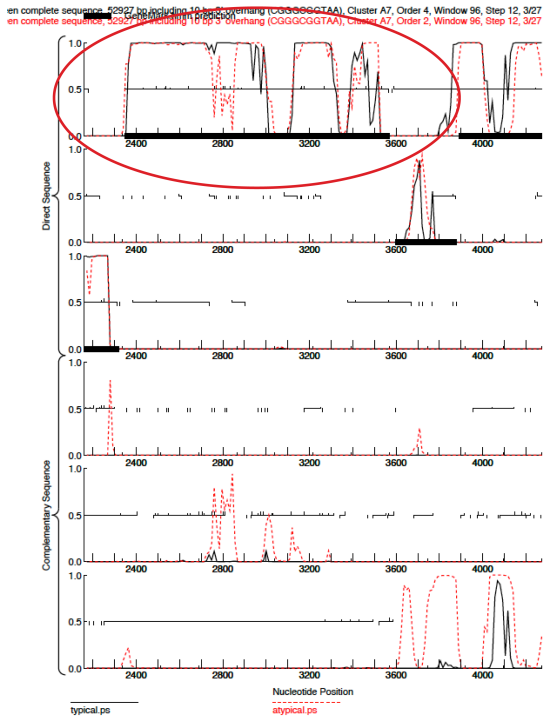


Figure 8.3

In **Figure 8.3**, the region of gene 5 is circled. You can find a gene by looking at its coordinates in the Feature Table, then finding those coordinates on the GeneMark output. Use the **stop coordinate** to identify the correct reading frame. For any given ORF, there may be many possible starts, but only one stop.

GeneMark (smeg) shows that this ORF has coding potential starting near position 2300+ and ending near 3600. You now have verified that all three coding prediction programs have predicted this gene. Strong evidence that you have a gene! Now you want to evaluate whether this is the best way to call the gene. Do you have the best start chosen?

How many other mycobacteriophages have this gene and do they all have the same start? Examine the BLAST data under the [[Blast]] sub-tab (**Figure 8.4**), and see if there are genes in the databases that are high-quality matches to this one.

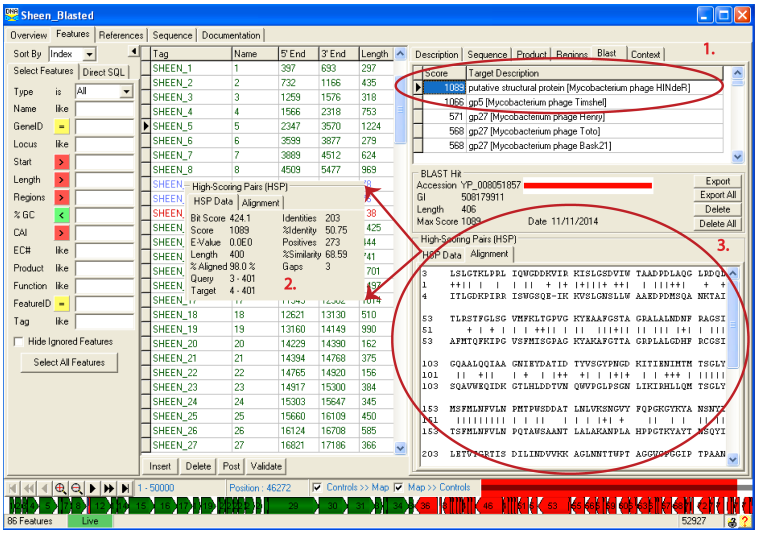


Figure 8.4

Note to Figure 8.4: The inserted box is obtained by clicking on the sub-tab **[[HSP data]].** DNA Master can display either HSP data or Alignment data at any one time.

1. The first match to Sheen's 5th gene under the parameters of BLASTp in DNA Master is to a putative structural gene in mycobacteriophage HINdeR.
2. Evaluate the E-Value. The score is 0.0E0, which is not exactly identical but close. (The E-value on NCBI's BLASTp (default parameters) is 1e-122). Why is that?
3. The HSP data describes it as 98.0% aligned, 50.75 % identity, and 68.59% similarity. See BLAST glossary (http://www.ncbi.nlm.nih.gov/books/NBK62051/) for definitions of terms.

Review gene length to make sure it meets the expected parameters. You will recall (see **Section 7.2**) that you should carefully examine genes less than 150 bp in length with an eye towards gauging their legitimacy, and genes below 120 bp should be viewed very skeptically. You can see gene length only when you are in the Widened Feature Table Mode (right click on **Name** at the top of the table. (see **Figure 8.5**), or you can select your gene and the length will be listed under the **[[Description]]** sub-tab to the right (see **Figure 8.5**). In this case, the gene length (1224 bp) is fine. Note that the amino acid length is 408.

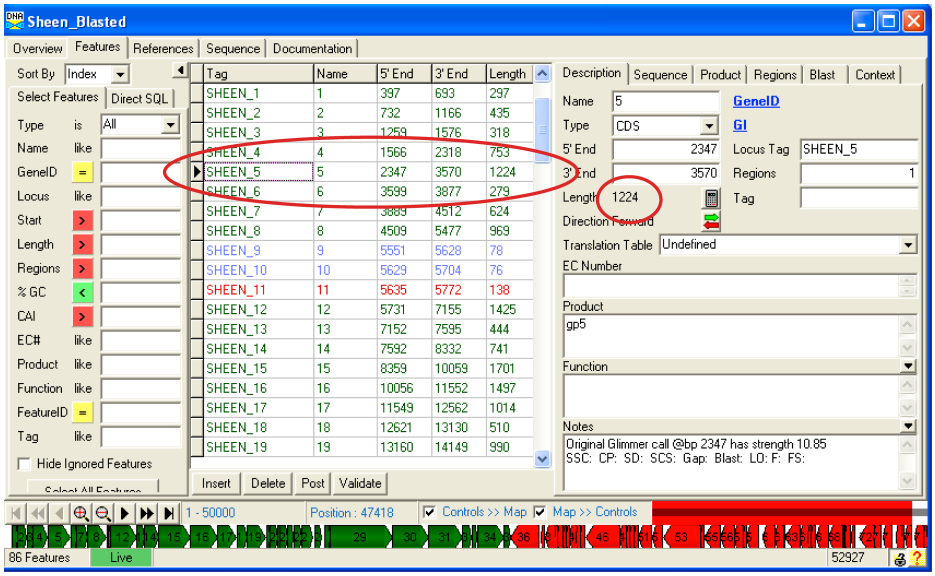


Figure 8.5

Verify that there is only one gene called in this region of DNA, as per Guiding Principle #1. The easiest way to do this is by viewing either the Phamerator map or DNA Master map you've generated to see if there are other genes called that substantially overlap this one on either strand.

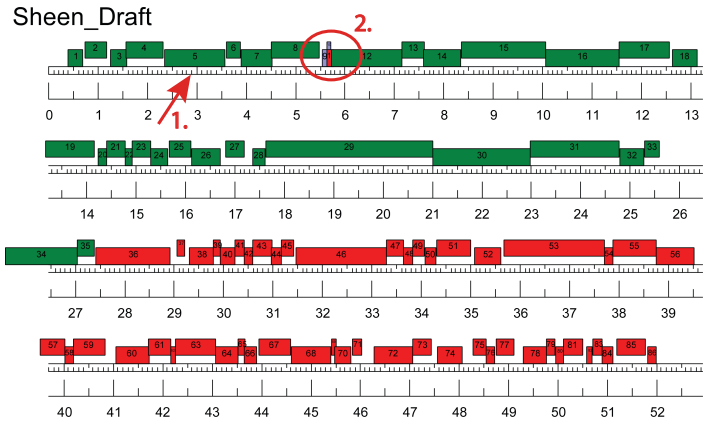


Figure 8.6

1. In this example above, we can see from the DNA Master generated map that there are no other genes occupying the same portion of DNA at gp5.
2. Note: Genes 9, 10 and 11 will need to be reconciled at a future point.

DECISION TIME: Is the designation of this ORF as a gene well-supported?	
GUIDANCE: Most gene calls will pass this stage. Exceptions are genes that are called by only one program, have little or no coding potential, have very weak or no BLAST matches, are too short, and/or substantially overlap other genes.	
YES **ACTION:** Continue to **Section 8.4.2**.	**NO** **ACTION:** You need to delete this gene. Go to **Section 9.2.1** for instructions.

8.4.2 Is the called start site for this gene the best possible choice?

This can be a tricky, but the simplest way to answer is to address the following questions.

Does the currently predicted start site include all of the coding potential in the GeneMark output? The current start position for our example is in location **A** in **Figure 8.7** below, and captures all of the coding potential. A hypothetical start at position **B**, however, would be a poor choice because it excludes coding potential.

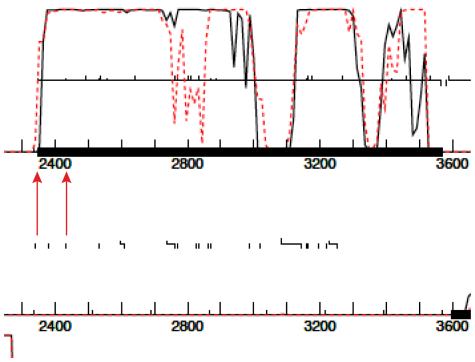


Figure 8.7

Did Glimmer and GeneMark agree on the start for this gene? Check the 'Notes' field under the **[Feature]** tab and the **[[Description]]** sub-tab to answer this question. In our example, shown in **Figure 8.8**, the two programs agree.

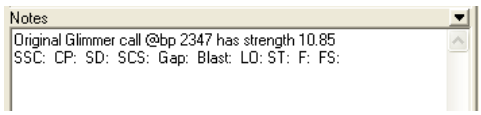


Figure 8.8

Is the predicted start codon the longest possible for the ORF without causing excessive overlap? The start codon, 2347, provides the longest gene possible for this ORF and does not overlap with the previous gene.

Does the start site match other starts for similar genes in GenBank and the mycobacteriophage database? To view the relevant information from NCBI BLAST, go to the **[[Blast]]** sub-tab, then the **[[[Alignment]]]** sub-sub-tab. You can select different BLAST alignments in the top pane to see how your start compares to those in a variety of other genomes. Refer to **Figure 8.7** for the alignment display. To review the relevant information from PhagesDB, select your protein and perform a BLASTp. Either and/or both will provide you with a global look at this protein and its homologues. Does it match proteins only found in the same subcluster or cluster of mycobacteriophages? Does it match phages from other clusters? Does it match phages of other species?

Remember to not be overly enthusiastic about alignment to other gene products, because you don't know *a priori* whether these were correctly identified. You just know that someone made that choice during a previous annotation. In addition, our example only has 68.59% similarity, 50.75% identity even though the 98% of the sequence aligns. It would be wise to look at the alignment across more than one homologue. The best approach to this is to use the two matches from the same subcluster, HINdeR and Timshel. The chosen start is the best alignment to those genes.

Using Starterator to evaluate gene starts. Sometimes the comparison with genes in a particular pham is what you will want to evaluate. You can use Starterator to evaluate that. Starterator provides you with alignment adta to evaluate the starts of a given Pham.

Refer to **Section 6.2** for how to run Starterator for your gene of interest, in this case Sheen, gene 37 (stop at 29071). From the Phamerator map, you can identify that this gene belongs to Pham 6183. The Starterator report has 3 components: the visual representation of each member of the Pham (represented as "Tracks"; a list of labels for each track; and the Recommend Start. As you start from the top and review coding potential, gaps, and BLASTP alignments, you will want to change the start of this gene (It does not capture all of the coding potential, doesn't fill the gap, or possess the best SD scoring.) You could run Starterator and evaluate its output because this output would show you if the start that you think is most appropriate has been called in other genomes. In this example (See **Figure 8.9**), the Starterator output confirms that the most upstream start is clearly a better choice.

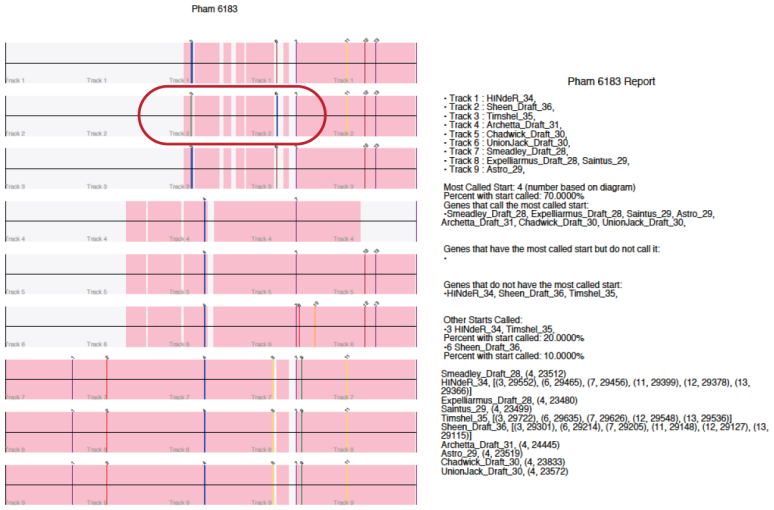


Figure 8.9

Other Starterator notations are "NA" for "Not Applicable"—for orphams, or for genes in which the evidence overwhelmingly supports a single start choice; or "SS" for "Suggested Start".

Does the predicted start have an associated ribosome binding site [RBS; Shine-Dalgarno (SD)] with a high score or recognizable sequence?

While ribosome binding site sequences can be found in phage genomes, the presence of a high scoring RBS is not the strongest determinant for what start to choose for any particular gene for a number of reasons. First, the most common arrangement of start and stop codons in phage genomes is a 4bp overlap between genes (for example, with the sequence ATGA: in which the –TGA is the stop of the upstream gene, and the ATG- is the start of the downstream gene). This arrangement allows for co-translation and does not require the ribosome to rebind to an RBS for the downstream gene. Second, leaderless transcripts (mRNAs that begin with the first base of the start codon) have been identified for some genes in phage genomes, suggesting that the ribosome is binding to the mRNA through some other mechanism that the SD sequence. Finally, ribosome binding is a mechanism for the regulation of protein expression, such that a poor binding site may be essential for limiting the levels of a protein made during infection. So while Shine-Dalgarno sites should be evaluated for every gene, the start with the best SD score may not be the best choice for a given gene's start.

The Shine-Dalgarno (SD) sequence in *E. coli* is AGGAGGA. It is located 7- 10 bases upstream of the start site. The Shine-Dalgarno sequence helps recruit the ribosome to the mRNA by aligning it with the start codon. Both the sequence and the spacing of the sequence are important in the evaluation. Dr. Lawrence recently rewrote the algorithms that underlie this evaluation using metrics described by Kibler (SD Scoring Matrix) and Karlin (Spacing Weight Matrix).

You will want to open the "Choose ORF start window to evaluate the ORF. (Check **Section 4.4.4** for details on how to open the "**Choose ORF start**" window.)

In the Choose ORF start window (See the oval of **Figure 8.10**), you must first select the SD scoring and Spacing Weight matrices. You will find multiple options (in a drop down menu) for both matrices. With Dr. Lawrence's recommendations and some trial and error, we suggest using Kibler 6 and Karlin Medium.

Figure 8.10 shows that there are 24 possible start codons for our example, gene 5 (coordinates 2347 – 3570) of Mycobacteriophage Sheen. For each start, this window displays, a raw SD score, Genomic Z value, spacer distance, final score, upstream sequence to that start (can you find an AGGAGGA-like sequence?), the start codon, start position, and ORF length.

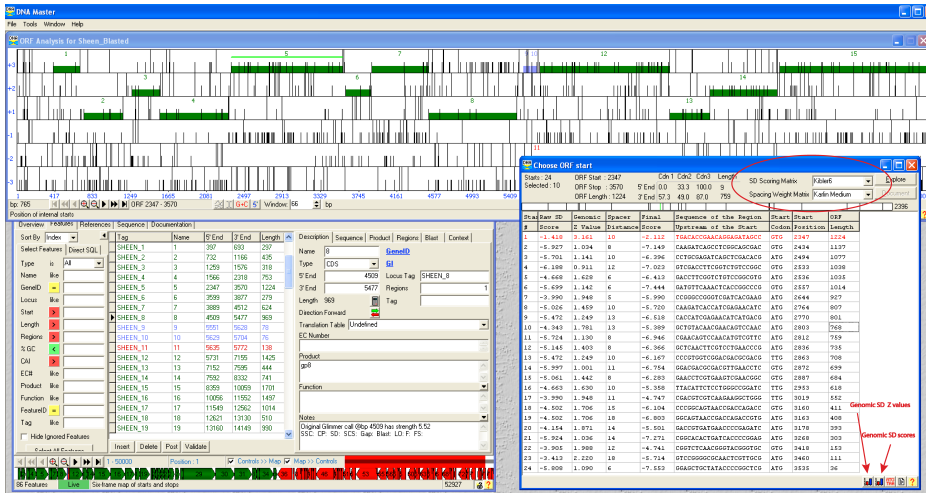


Figure 8.10

Use the following to best evaluate the information in this window:

1. You are provided the Raw Score (how well the SD sequence matches) in addition to the spacing and the final score. This allows one to assess the match independent of the spacing. The higher the score (the less negative), the better the sequence match. This Raw Score compares the SD of the start of interest with all of the SD scores across the genome (a distribution of scores). This scoring system is based on log probability scores. They are negative numbers and higher (less negative) are better. Moreover, magnitude is now meaningful. A score of -2 is ten times more likely than -3.
2. You can see that distribution by clicking on the Genomic SD score button in the bottom right of the choose start window. (See arrow in **Figure 8.10**.) To evaluate the distributions, change the bin counts to 20. If you see a normal distribution, proceed.
3. The normal distribution of the scores will be mostly random. These are ALL SD sequences, even in frames where no genes are predicted. So you are looking at the scores with the highest value (least negative number). However, the scores you see in your list are NOT random, but remember all but one are not correct!
4. The distribution of scores throughout the genome is used to provide a Z-value for each raw score. You can see the distribution of Z scores by clicking on the Genomic SD Z value button, and changing the bin count to 20. Z is the number of standard deviations from the mean. A Z-value higher than 2 is getting good.
5. The spacer distance is the number of bp this sequence is found upstream of the start. You will recall that you want the number to be between 7- 10.
6. The final score is determined by both the raw score and the spacing. It is evaluated in the same way as the raw score, where the higher the score (the less negative), the better the sequence match.

Note: The settings for this window are open for exploration. In general, when this particular gene is not controlled by a Shine Dalgarno sequence, this evaluation should not provide a clear answer. Further evaluation on this point is needed.

Applying this to our example, the start 2347 has the highest Raw SD Score and has a spacer distance of the predicted length (10). It is the highest final score (-2.112) and is at least 100 times more likely than the next lowest score (-4.714). The Z-value of 3.161 is the best in the list.

Note: The old DNA Master score is still available, but deprecated. It will eventually be removed. No Z-values, base distributions or probabilities are available in that format.

You now need to put this information together to make the best choice and record your decision in the notes.

In this example, the initial information in the Notes window is:

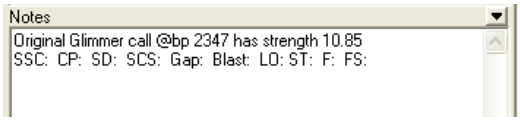


Figure 8.11

To complete the notes for gp 2, you can enter something like this:

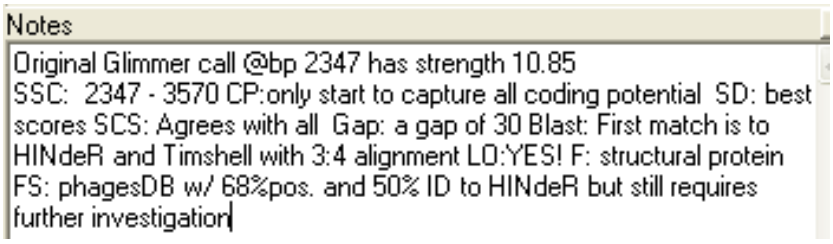


Figure 8.12

SSC: Record actual coordinates. 2347-3570. This may seem redundant, but this points to a common oversight. As you make decisions to change a start, you forget to actually change the start in the Feature Table (**Section 9.2.3**)

SD: Does this call have a relevant SD and is it the highest score?

SCS: This gene was called by all coding potential prediction programs with the same start.

Gap: Yes, there is 30 bp gap that cannot be filled with a coding potential prediction. This points in the direction that there may be a ribosomal binding site upstream of this gene.

Blast: The first hit is to Mycobacteriophage HINdeR. BP 3 of Sheen aligns with BP1 of HINdeR.

LO: Is this the Longest gene possible in this ORF? Yes

ST: Starterator data (where applicable). (Enter NA when it is not applicable---as in the case for an orpham with no close matches, or NI if Starterator was run but is Not Informative.)

F: and FS: Refer to Section 10 to complete this part of the gene evaluation. In this example, "structural protein" was noted in the BLASTp at PhagesDB.

For complete instruction on documenting gene calls, refer to **Section 9.6.**

DECISION TIME: Is the currently called start site for the gene the best choice?	
GUIDANCE: Ten percent or more of your genome's start sites will likely have to be changed, and in some cases NEITHER Glimmer nor GeneMark will call the correct start. For each gene, gather the information described in this sub-section, and try to weigh all possibilities to arrive at the best call.	
YES **ACTION:** Continue to **Section 8.5.**	**NO** **ACTION:** You need to change this gene's start. Go to **Section 9.2.3** for instructions.

8.5 Checking gaps in the draft annotation for uncalled genes

According to Guiding Principle #3, the genes in phage genomes are generally tightly packed, so any large gaps (>50 bp) in your annotation should be reviewed.

In circumstances where you have a series of genes in the same orientation that are likely to be expressed as an operon, these genes are typically nestled closely end-to-end. However, non-coding gaps are perfectly legitimate and to be expected, and filling gaps with poorly justified gene calls is not appropriate.

There are two basic things you should look for in gaps.

Can the start site of the downstream gene be extended so that the gene covers more of the gap? Carefully consider all possible start sites for the downstream gene. If a longer one is available, compare it to the current start site to see if it is a similar or better choice. All other things being equal, a longer call is usually preferable, but do not extend genes just to fill a gap.

If YES, go to Section 9.2.3 to change the start site.

Is there a protein-coding gene in this gap? You have several resources to help answer this question. First, you can use Phamerator maps to see if any similar genomes have a gene called in this gap. Second, you can look at the GeneMark-Smeg output to see if any of the reading frames in this gap show some coding potential. Third, you can copy the DNA sequence from your gap and use it to run a BLASTX search on NCBI. The combination of these techniques may yield convincing evidence that the gap contains a protein-coding gene that was missed by both Glimmer and GeneMark.

If YES, go to Section 9.2.2 to add a gene.

Remember too that you should expect non-coding gaps between divergently transcribed genes as there is a strong prediction that promoters lie within these regions. For example, in **Figure 8.13**, we should expect some gap between gene 47 (transcribed leftwards) and gene 48 (transcribed rightwards).

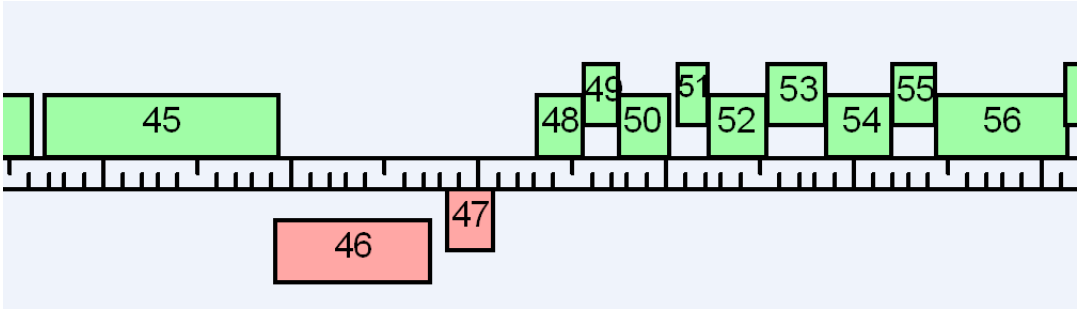


Figure 8.13

8.6 Finding and refining tRNA and tmRNA genes

DNA Master searches for tRNAs by default, but may miss some tRNAs that other approaches can find, or may miscall the precise boundaries of these genes. See **Section 9.5** for information on how to search for and call tRNAs and tmRNAs.

8.7 Completing your annotation refinement

Much of the work of annotation is following the steps above—for each gene and gap in your genome—until you've settled on the best calls for each with the information given.

As a double-check, you should scroll through the Feature table and the genome map (using buttons at the bottom of the **[Feature]** tab) to make sure that all the changes you've made have been committed to the file.

One suggestion to confirm that the changes you have made are actually in your file is to use the Genome Comparison Tool in DNA Master. (See Protocols -> Further Discovery -> Exploring Bacteriophage Biology). As you make changes in your file, the gene calls can become disparate with the auto-annotation found in Phamerator. The Genome Comparison Tool produces a quick, easily produced genome map compared to any genome(s) of your choice.

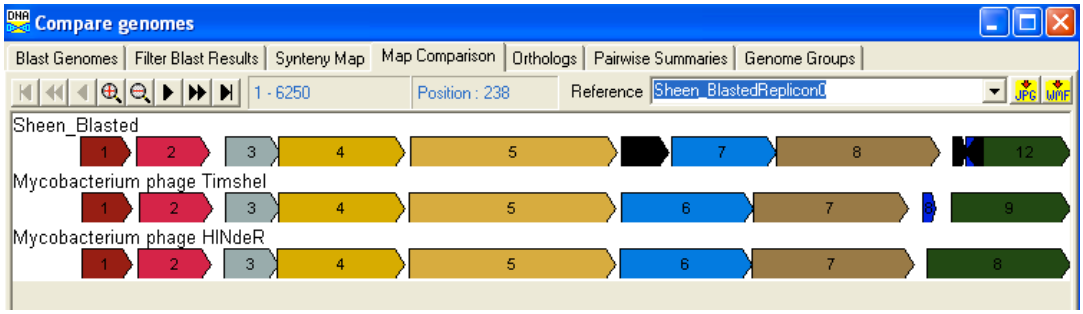


Figure 8.14

Several important steps remain.

1. **Documenting your gene calls**. You can use the Notes field (under **[Feature] [[Description]]**) to record notes about each gene as you go. Your final submitted file, however, should have each gene's Notes field filled in according to specific instructions so as to facilitate checking the annotation. These documenting instructions are described in **Section 9.6**.

2. **Determining putative functions**. You've figured out where the genes are (and aren't), so the next step is to see if you can make a well-supported guess as to what they do. This process is covered in **Section 10**.
3. **Merging several different portions of the annotation into a single file**. In a classroom setting, often you will choose to split the genome into sections and have different groups or students work on different sections. If you've split the genome up, now is the time to bring everyone's work back together or "**Merge**" the different annotations. This process is described in the first part of **Section 11**. We recommend that you make this a vibrant part of your classroom structure. It can be quite problematic to think that you will do all the 'checking' work out of class.
4. **Checking the final annotation**. Once you've produced a nearly final annotation, it still needs a (relatively) expert eye to double-check it, as described in **Section 11**.
5. **Submitting final files**. When you're confident in your annotation, have investigated every nook and cranny, and are ready to send it out the door, you'll need to generate and submit a final DNA Master file, as well as a list of those who have worked on the annotation and should be authors on the GenBank submission. This is described in **Section 12.**

9 The mechanics of making changes to your annotation

9.1 Overview

This section, unlike most sections of this guide, is not intended to be a sequential step-by-step description of any part of the annotation process. Rather, it is intended to be used as a reference section for how to make specific changes to your annotation. The actual decision-making steps were described in **Section 8**, and a graphical summary can be seen in the Decision Tree in **Section 8.3**.

The three most common operations you'll need are covered first. They are:

- Deleting a gene
- Adding a gene
- Changing the start site for a gene

The following sub-sections describe some common steps you should take after making any changes to your annotation. They are:

- Posting changes
- Validating your calls
- Renumbering your genes
- Re-BLASTing a gene you've changed

There are also some less common operations that you may need. They are:

- Annotating a programmed translational frameshift
- Annotating introns
- Annotating wrap-around genes

Next is a sub-section on RNA genes. It is:

- Predicting tRNA and tmRNA genes

Finally, there is a sub-section of how to document the annotation work you've done:

- Documenting your gene calls

9.2 Making common changes to your annotation

9.2.1 Deleting a gene

- Select the **[Feature]** tab of your main genome file.
- In the center column, click on the feature you would like to delete to select it. (The selection can be verified by the presence of a black arrow to the left of the gene name.)
- Click the '**Delete**' button, found at the bottom of the center column.

- Click the '**Post**' button to commit your changes to the database.

9.2.2 Adding a gene

If it's not already open, open the Frames window by going to **DNA → Frames**

- Locate the ORF that corresponds to the gene you would like to add.
- Click within that ORF, and a green or red line will appear, depending on its orientation.
- Click on the '**RBS**' button in the lower-right corner.
- Confirm that you have selected the correct frame by verifying the coordinate of the **STOP** codon. There can be many possible starts for each ORF, but there is only one possible stop!
- Choose the best start, as described in **Section 8.4.2**, then click anywhere in that start site's row in the "Choose ORF start" window to select it.
- Return to the **[Feature]** tab and click on the '**Insert**' button at the bottom of the center column.
- A new window will appear that allows you to add the feature. Verify that the correct orientation (forward/reverse) is selected and that the coordinates are correct. Do not worry about adding the correct gene number or gene product (gp) number, as the genes will get renumbered using the Validation function when you are done.
- Check the boxes '**add to feature table**' and '**add to documentation**'.
- Click '**Add Feature**'.
- Click the '**Post**' button to commit your changes to the database. This is also a good time to save your file.
- Your new gene will likely be placed at the end of your feature list, because the default sorting is by index number, rather than genome position. To sort by position, find the dropdown box at the top left of the **[Feature]** tab labeled '**Sort by**', and change it from "Index" to "Start."
- You may want to collect BLAST data for your new gene. See **Section 9.3.4** for instructions.

9.2.3 Changing the start site for a gene

- Select the **[Feature]** tab of your main genome file.
- In the center column, click on the gene you want to change to select it.
- Click on the **[[Description]]** sub-tab to the right.
- In the box labeled "Start", third from the top under "Description", type in the new start coordinate you've selected.
- Click on the Calculator button (this is an icon of a calculator, found just to the right of the "Length" display) to recalculate the ORF length. The new length (in bp) will be shown and should reflect your change.

- Click the '**Post**' button at the bottom of the central column to ensure your changes are saved to the database. This is also a good time to save your file.
- Because you've changed the start site, you'll probably want to re-BLAST this gene so that the BLAST results reflect your change. See **Section 9.3.4** to do so.

9.3 Common steps to take after making changes

9.3.1 Posting changes

When making gene changes—including changing start codons, deleting genes, annotating programmed frameshifts, adding notes to the Notes field, etc.—you need to both **enter** and **post** the changes. Simply entering them is insufficient, and the changes may be lost. Once you've learned how to post, it doesn't hurt to **post often**!

Normally, a selected gene in the feature table will be indicated by a triangle, as shown below.

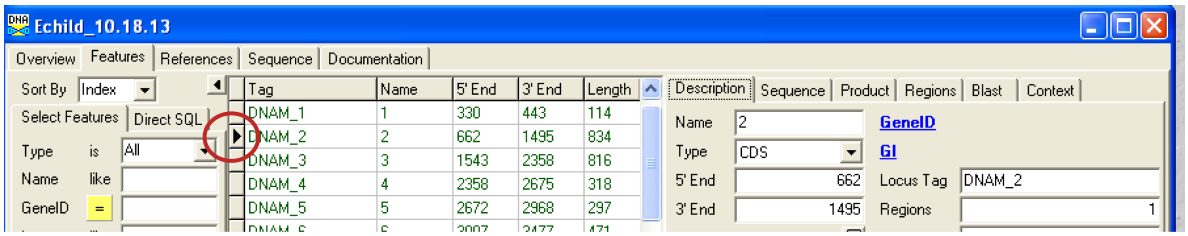


Figure 9.1

When you make a change to a feature listed in the Feature table (e.g., begin typing in the Notes field), the icon next to the feature changes to an Insert icon, as shown below.

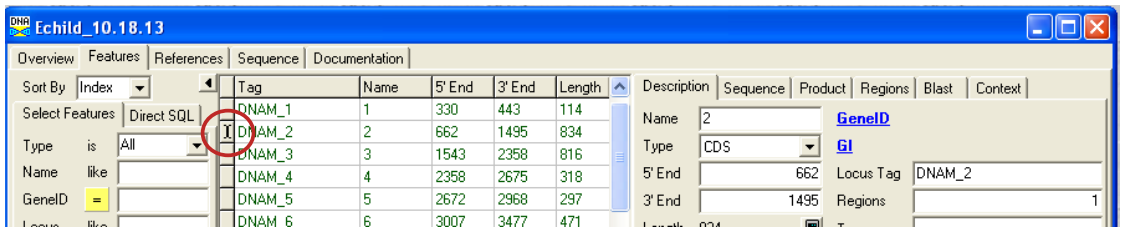


Figure 9.2

For the most part, this change to Insert Mode happens automatically when you start typing in any of the fields under the Description tab. Your changes , however, **won't be posted to the database until you exit Insert Mode**.

The following are ways to make sure your edits get posted to the database.

- Click on the '**Post**' button at the bottom of the center column.
- Click on the **Calculator** icon, after changing a start or stop.
- Click on a different feature in the center column.

You will be able to tell that your changes have posted to the database because the Insert icon will change back to the right-pointing triangle.

Important Note: The following are ways that your changes will **not be posted** to the database, and **WILL BE LOST**.

- Saving your file while still in Insert Mode.
- Clicking on a different tab or sub-tab while still in Insert Mode.

9.3.2 Validating your annotation

As you work through your genome, DNA Master has a handy **validate** feature that helps ensure your gene calls have valid start/stop codons and do not have any internal stop codons.

To perform a genome validation, follow the steps below.

- Click on the '**Validate**' button, at the bottom of the central column in the **[Features]** tab (located in the red circle in **Figure 9.3** below).

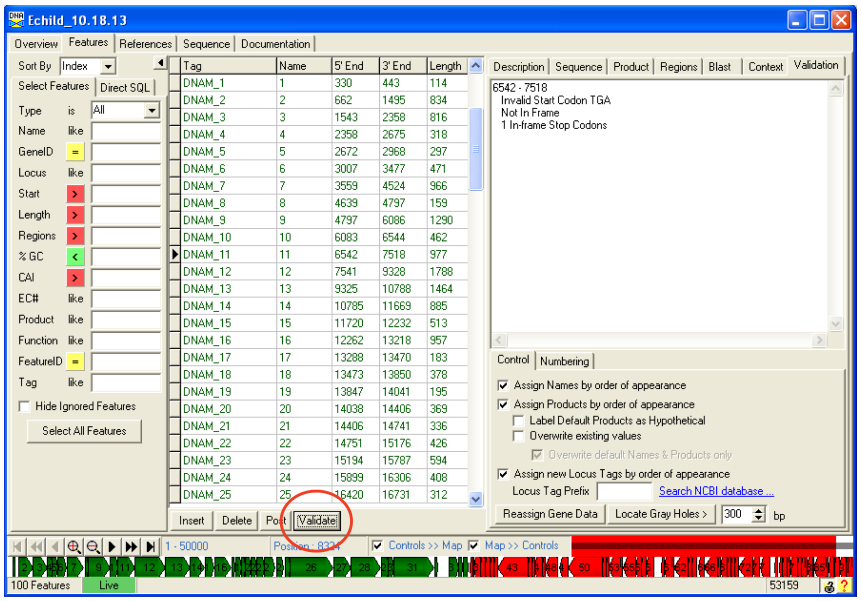


Figure 9.3

DNA Master will let you know when gene calls are not in frame or if they have incorrect start or stop codons. A genome is not complete unless validation returns as "All ORFs are valid".

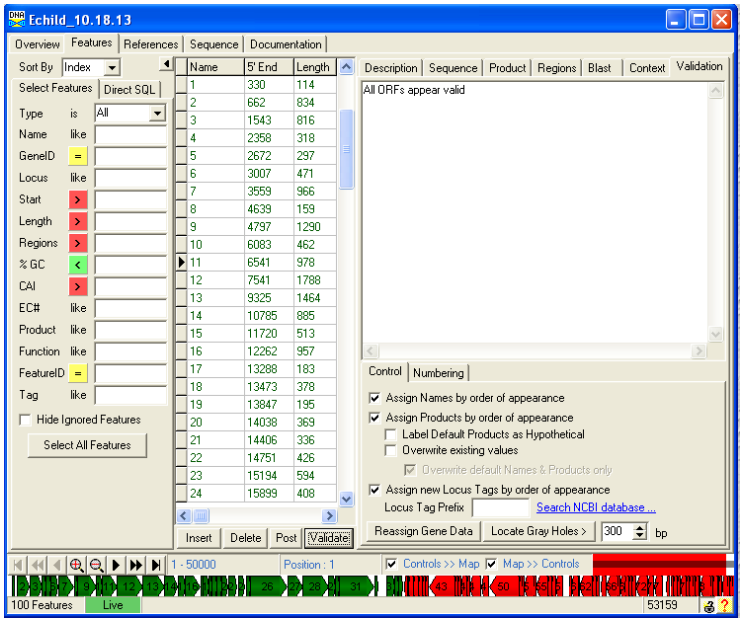


Figure 9.4

If the validation generates failures, you should check the coordinates in those features to see what might have gone wrong and make necessary changes. You can then re-run the validation to ensure all ORFs are valid.

9.3.3 Renumbering & formatting annotated features

When you add or delete a gene, you may want to renumber the genes to reflect the change. Genes added manually after auto-annotation will appear at the bottom of the feature list when sorted by **Index**. Sorting by **Start** will place the gene in its correct order by start coordinate.

To renumber your features:

- In the **[Features]** tab, click the '**Validate**' button located at the bottom of the central column. This will open the **[[Validation]]** sub-tab on the right side.
- Check the boxes as shown in **Figure 9.5**.
- In the field marked "Locus Tag Prefix", type in your phage's name (it will be all capital letters). This is a necessary attribute of each feature for a GenBank submission. We are adding it here so that 'Tags' are renamed in the same manner as 'Names' and 'Products'. For returning users, we are changing the format this year.
- Click the '**Reassign Gene Data**' button.
- Click '**Yes**' to confirm in the window that pops up.
- Genes will now be re-numbered sequentially.

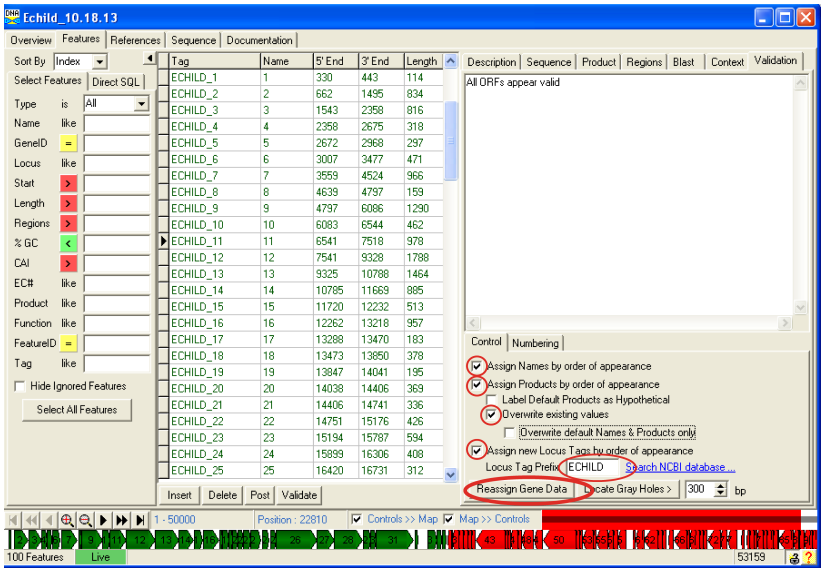


Figure 9.5

Note: If you're annotating a portion of a genome as one part of a larger group, you may not want to renumber genes because this may cause confusion if some groups do so and others do not. Make your own decisions, but bear this in mind. You can re-number as often or as little as you like.

Additional Note: Remember that the auto-annotation is based on a random sample of the genome. This means that all auto-annotations will NOT be identical. Therefore, your auto-annotation may not exactly match what was loaded into Phamerator. As you re-number, the gene numbers may again be modified from what is in Phamerator. It is prudent to identify gene by their STOP coordinate. Until a genome is published using gene names (in our case, numbers) all other means of identifying a gene a can lead to confusion.

9.3.4 Re-BLASTing a gene

Once you have finished adding a gene, changing a gene's start site, or entering multiple regions for a gene, it can be useful to re-BLAST the gene. This is particularly helpful to check whether or not a gene's modified start site now matches those published in GenBank.

- From the **[Features]** tab, select the **[[Blast]]** sub-tab.
- Click the '**Delete All**' button, identified in **Figure 9.6**. Do not skip this step. DNA Master does not overwrite Blast hits, storing multiple copies that can be confusing.

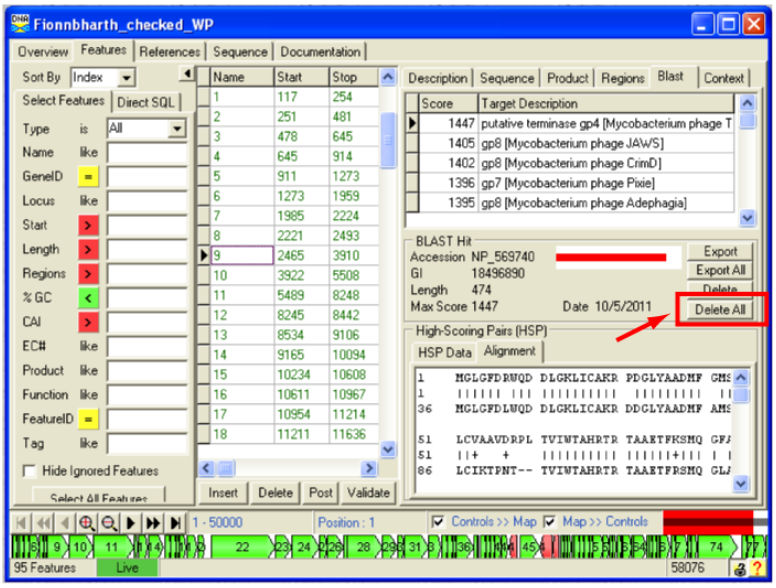


Figure 9.6

- A dialog box will pop up and ask if you really want to delete all the BLAST hits for this gene. Click '**Yes**'. The BLAST tab will now be empty of hits, as shown below.

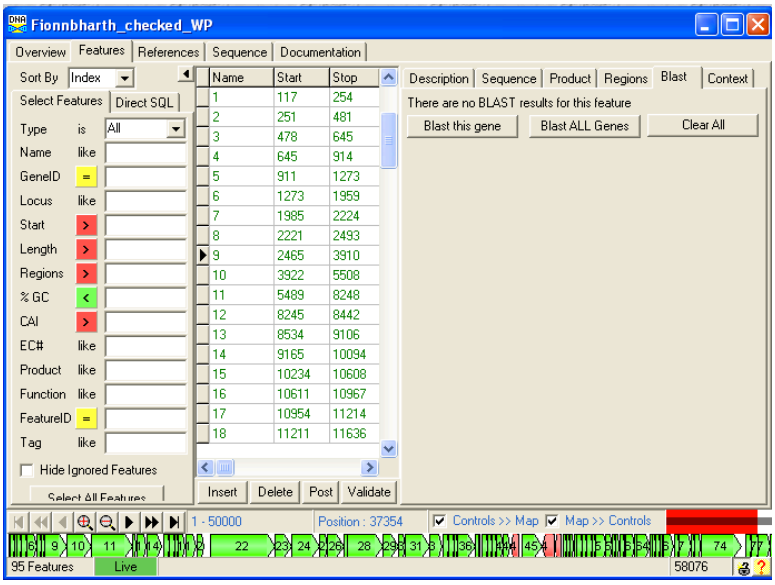


Figure 9.7

- Click the '**Blast this gene**' button.
- A new window will appear, labeled "**BLAST search for** [your gene coordinates]". The status of the BLAST attempt will continually be updated in this window until the BLAST is done. When it is finished, the window will display the BLAST results as shown in **Figure 9.8**.
- If you have not BLASTed all genes, or want to re-BLAST all genes you can do it here also.

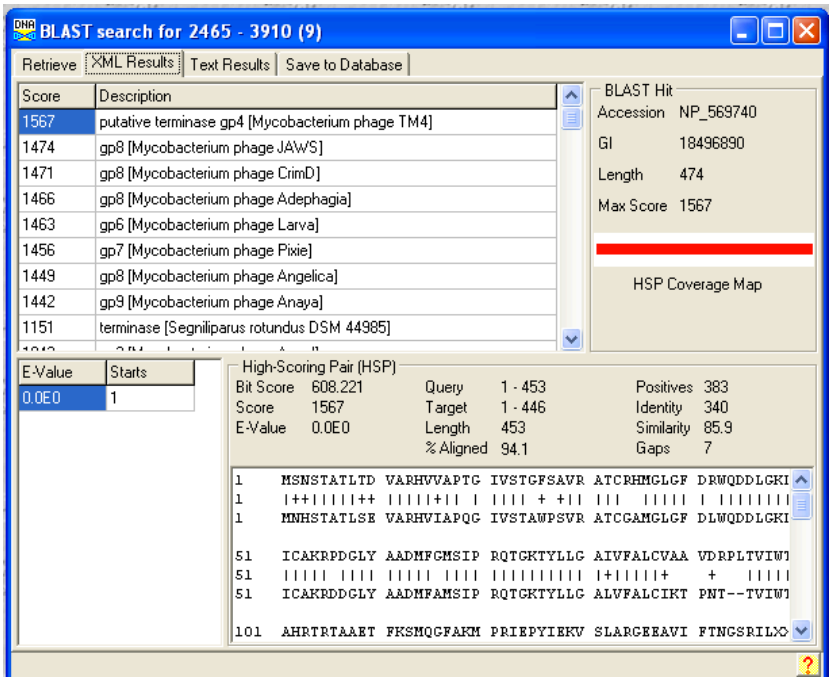


Figure 9.8

- To save your new BLAST hits to your genome file, select the **[Save to Database]** tab.

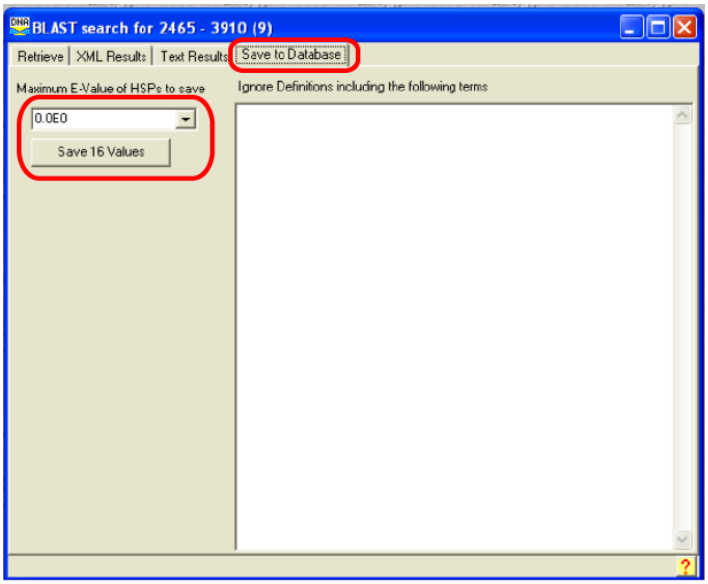


Figure 9.9

- Click on the drop-down arrow next to the empty field under '**Maximum E-Value of HSPs to save**'.
- Scroll through the listed E-values (these are from your new BLAST matches) and pick an appropriate value (greater than 10^{-3}) that also gives you a useful number of matches (at least 10 or so). If you only have E-values higher than 10^{-3}, just pick at least one match so you will know that you have BLASTed this gene, and it doesn't have any good matches in GenBank.

- Click the '**Save [n] Values**' button. The "n" will be automatically filled in for you based on the number of matches you picked from the drop-down menu. It should then say "**[n] saved**" in this window under the button. Close the BLAST window.
- Now your new BLAST hits should be listed in your genome file (you may not see them until you select a different feature and then reselect the one you just BLASTed to refresh the view).

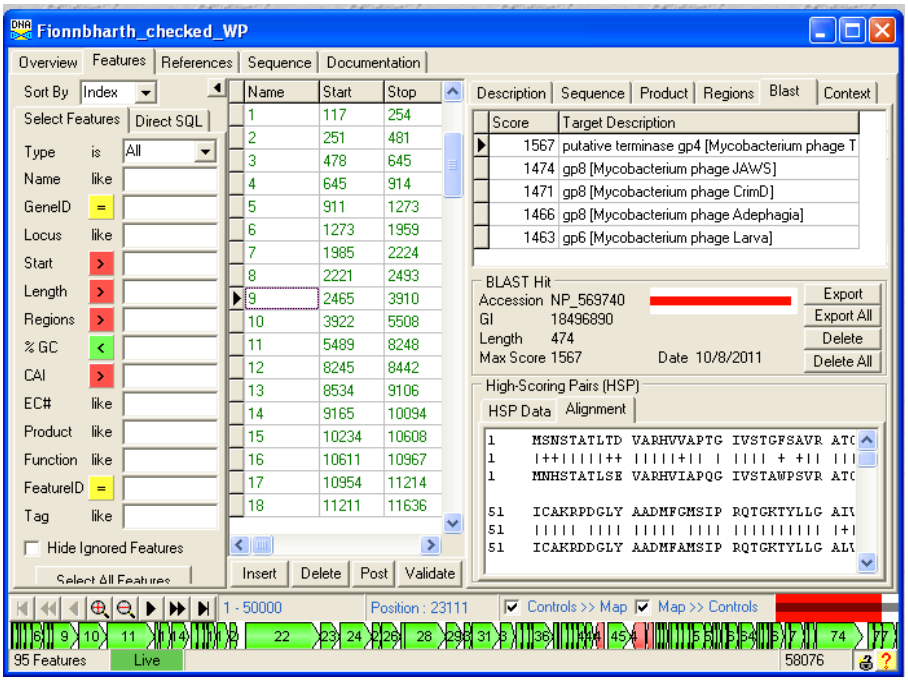


Figure 9.10

9.4 Making less common changes to your annotation

9.4.1 Annotating programmed translational frameshifts

Assuming you have identified the two genes involved in the frameshift (see **Section 8.4.3**), the next critical piece of correctly annotating a frameshift is locating the precise position where the shift occurs. A printed six-frame translation of the region in question is helpful during this process (see **Section 5.1**).

Frameshifting occurs when the ribosome encounters a "slippery" sequence in the mRNA, such as GGAAAA, and loses track of how to count to three. In the most common shift, the -1 shift, the first "A" of the above sequence is "counted" twice; it is read as the third nucleotide in the last codon of the upstream region, AND the first nucleotide in the first codon of the downstream region. (There are also examples of +1 shifts, in which a nucleotide is skipped, or -2 shifts, in which two nucleotides are counted twice.)

For those unfamiliar with finding the slippery sequences and determining where and how the shift is occurring, it is probably easiest to examine a similar phage's genome in Phamerator that has a correctly annotated fusion gene, and compare it to the six-frame translation of your own phage's fusion gene. This will help to determine what the correct amino acid sequence should be, and therefore which nucleotide the shift must occur at.

To annotate a programmed translational frameshift within your phage, you should do the following (we use Fionnbharth below).

Determine the precise location of the shift

- Using Phamerator or BLAST, find the most similar genome you can that has a correctly annotated frameshift. For Fionnbharth, we've selected Angelica.
- Make a Phamerator map using your genome plus the similar genome you've chosen (see **Section 6.4**).
- Click on the first gene in the correctly called frameshift in Phamerator to select it. Its border will change from black to orange to indicate that it's selected, and its nucleotide and amino acid sequences will be displayed in the panels at the bottom of the window, as shown in **Figure 9.11**.

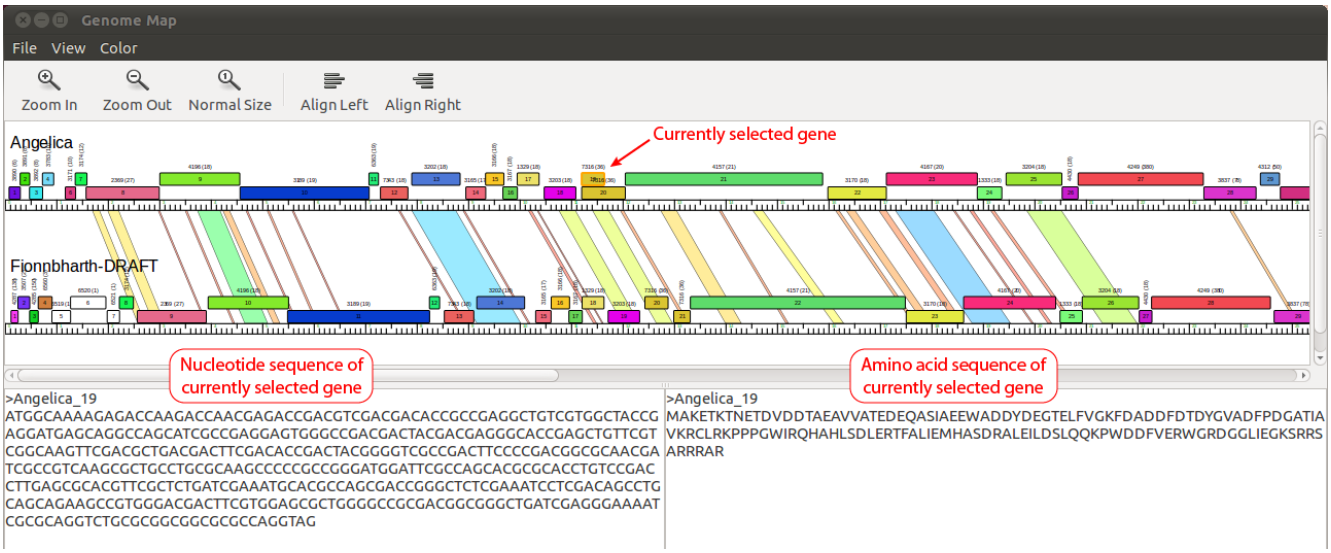


Figure 9.11

- Copy the amino acid sequence from the bottom-right panel and paste it into a new text file.
- Now select the second correctly called frameshift gene (just below the first), and copy and paste its amino acid sequence into a new text file as well.
- Locate the precise position where these two amino acid sequences diverge. (This can be done by manual inspection of the amino acid sequences, or by using BLASTP with the "Align two or more sequences" option checked.) In our example, the two Angelica sequences diverge after amino acid 135, as shown:

  … GGLIEGK**SRRSA…** in the first protein.

  … GGLIEGK**IAQVC…** in the second (fusion) protein.

- Now back to your genome. An examination of your six-frame translation shows the two genes as they were called by DNA Master's Auto-Annotate function.

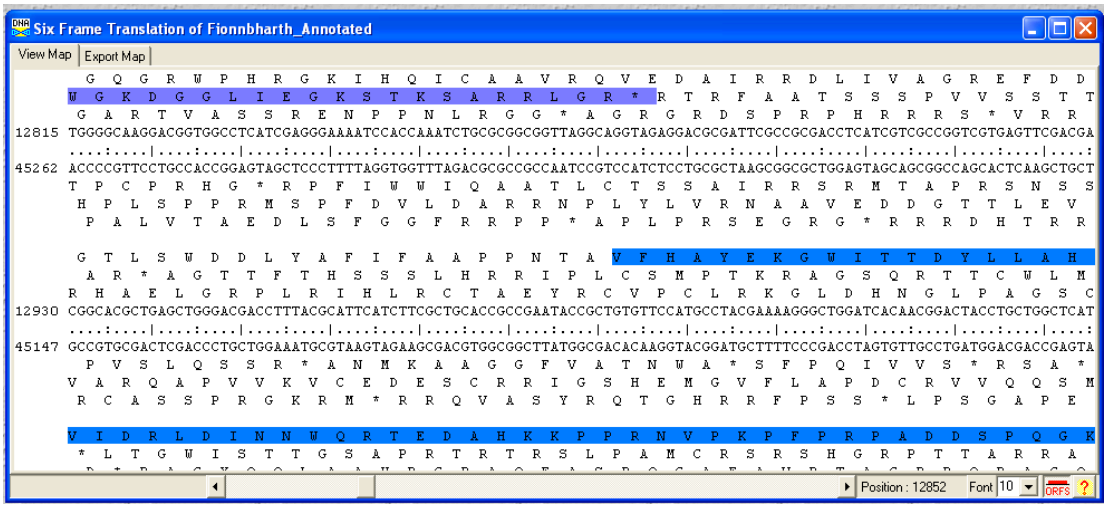


Figure 9.12

- In **Figure 9.12**, the purple bar shows the end of the first protein, and the blue bar shows the beginning of the auto-annotated version of the second protein. Note that the purple highlight is in reading frame 2 while the blue is in reading frame 1. This means that this phage likely has a -1 frameshift, and we need to identify a nucleotide somewhere in this region that should be "counted" twice by the ribosome.
- Near position 12841 there is an obvious slippery sequence, "GGGAAAA" (underlined in red below). If we count the first A (at position 12844) of this sequence twice, we shift frames as shown by the red box, and generate the amino acid sequence …GGLIEGK**IHQIC**… in the fusion protein. This sequence is not identical to Angelica's fusion sequence, but it is very close. Counting carefully from the left, we can determine that the first "A" at position 12844 (underlined in green) is the coordinate of our frameshift.

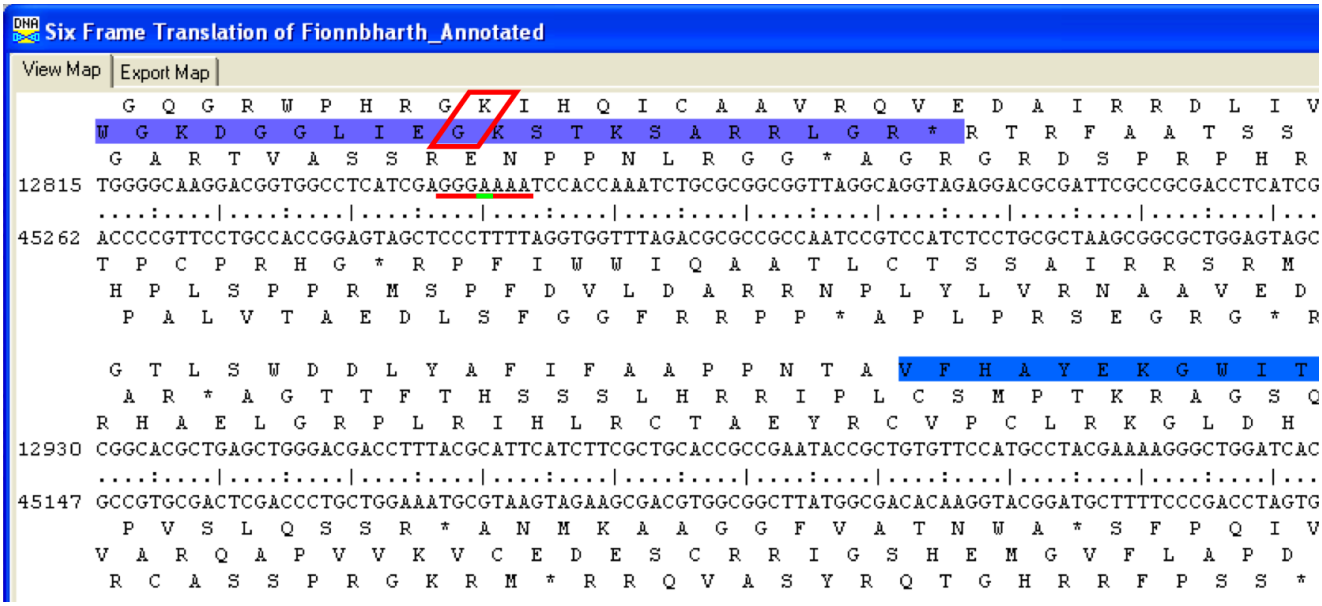


Figure 9.13

Annotate the frameshift in DNA Master

- Go to the **[Features]** tab and click on the **second** of the two genes involved in the frameshift. (We do not need to modify the first gene, only the second.)
- In the **[[Description]]** sub-tab in the right-hand section, locate the field labeled "Regions" (far right column, shown below). Change the number from "1" to "2", then click the '**Post**' button at the bottom of the central column to save this change.

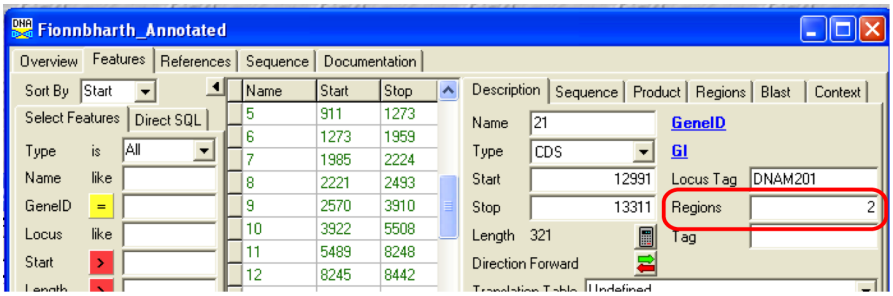


Figure 9.14

- Change from the **[[Description]]** sub-tab to the **[[Regions]]** sub-tab in the right-hand section of the Features tab.
- You will now enter the two regions that constitute the fusion protein. **These must be entered in order**, upstream first and downstream second.
- The **Start** coordinate for the **first region** is the start of the whole frameshift region (same as the start for the previous gene). The **Stop** coordinate for the first region is the position you've identified where the frameshift occurs; in our example it is 12844. For the **Length** field, just enter the number 1, because DNA Master will calculate this for us automatically in the following steps, but does require that some number be entered as a placeholder until then.

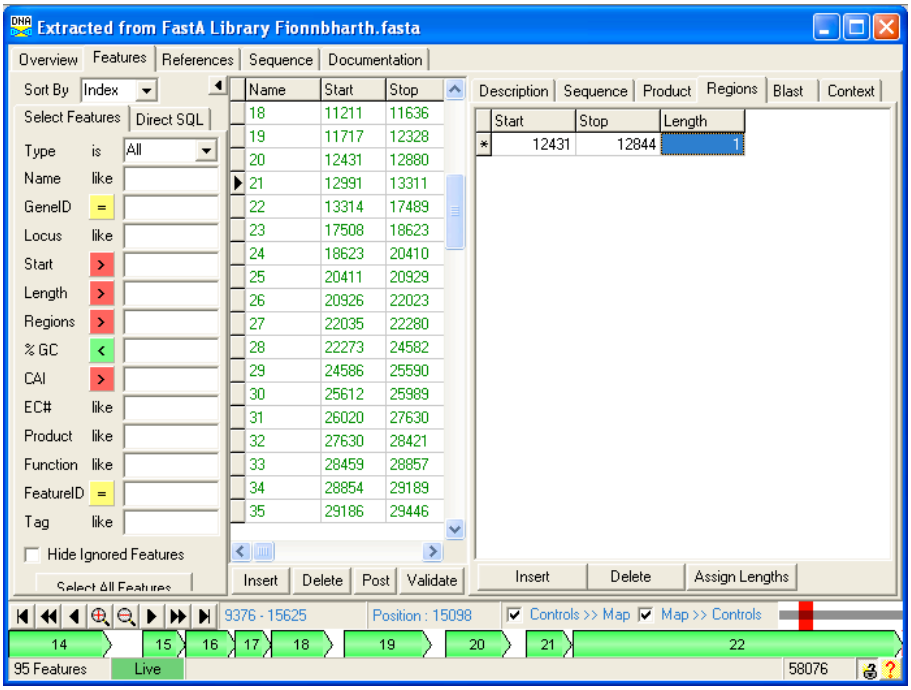


Figure 9.15

- With the "Length" field selected (as shown in **Figure 9.15** by the blue highlight), press **Tab** to move to the second line. For the **second region** of the fusion protein, the **Start** coordinate is the position of our frameshift (again, in our example this is 12844). The **Stop** coordinate is the previously called stop for the second gene (the end of the entire frameshift region, in our example 13311). Again, the **Length** should be entered as "1" for now.
- Click the '**Assign Lengths**' button at the bottom of the **[[Regions]]** sub-tab (see below). DNA Master will calculate the length of each region and display it in the "Length" column.

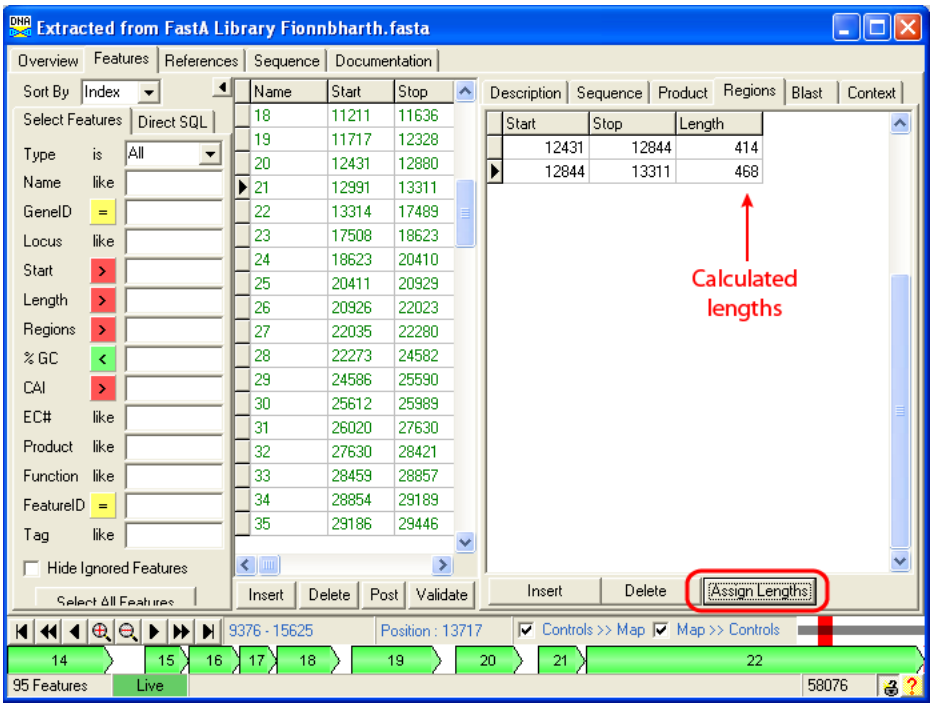


Figure 9.16

- Finally, change back to the **[[Description]]** sub-tab, and enter the correct start and stop coordinates for the entire gene (both regions). In our example, these coordinates are 12431 and 13311. Then click the **Calculator** icon to post changes and calculate the length of the entire gene.

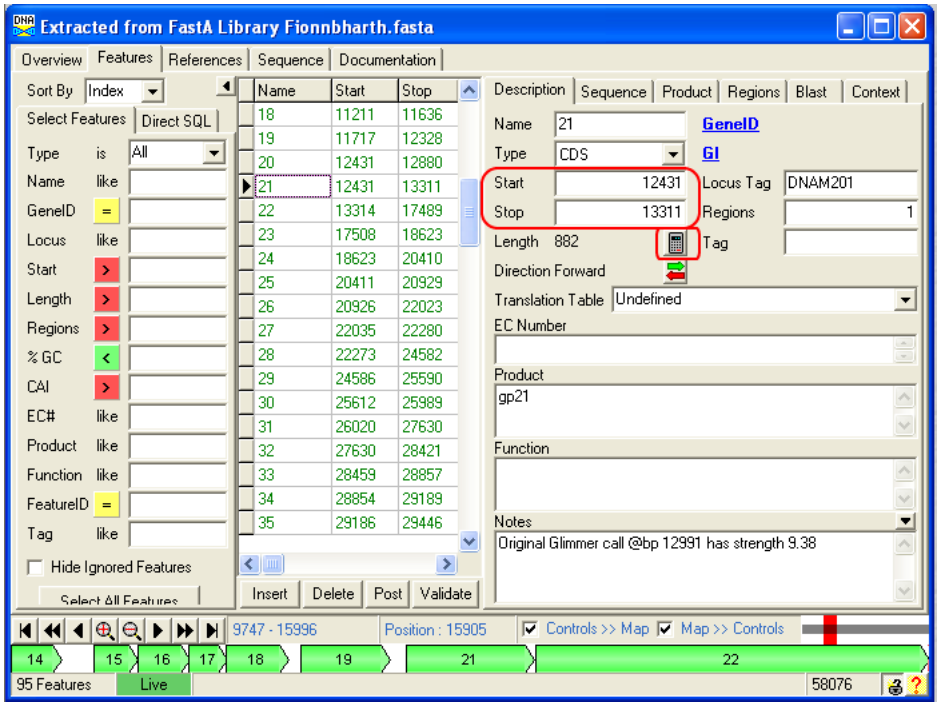


Figure 9.17

Now if you change back to the **[[Regions]]** sub-tab, you will see a graphic representation of your two frameshifted regions in black bars at the bottom of the tab, as shown in **Figure 9.18**. (You may need to select a different feature, then come back to this one to refresh the view.)

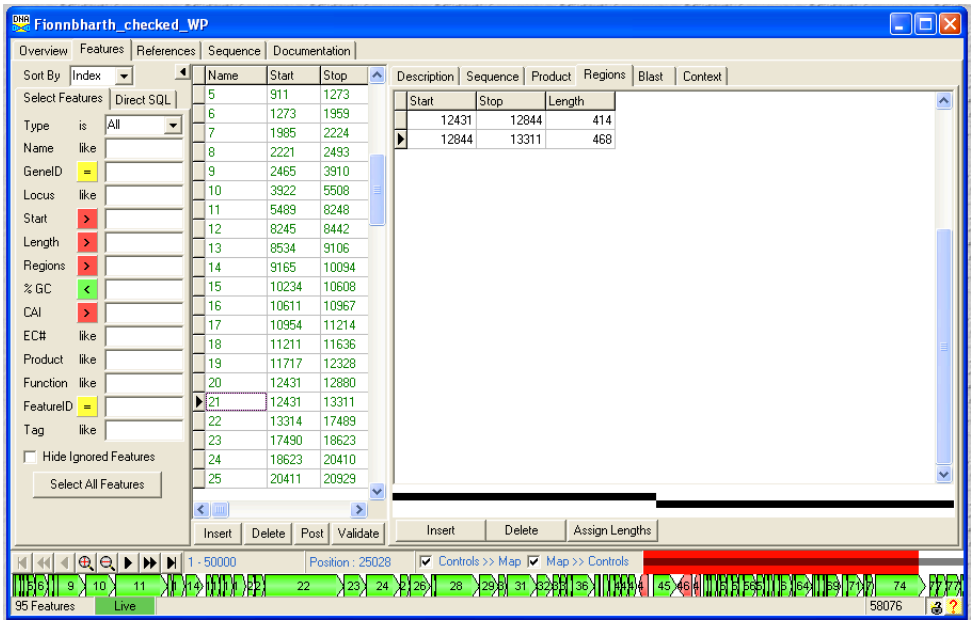


Figure 9.18

Note: The frameshift described here is a -1 programmed frameshift. Not all tail assembly chaperone frameshifts are -1. They can also be +1 (typical of Cluster F1 genomes) or -2. (Xu, J., Hendrix R.W., Duda, R.L. (2004) Conserved translational Frameshift in dsDNA Bacteriophage Tail Assembly Genes. Molecular Cell 16, 11-21.

9.4.2 Annotating introns

Genes with introns in them can be annotated as two regions by following the procedure above under the heading "**Annotate the Frameshift in DNA Master**." In this case, the two regions you enter will correspond to the exon portions of the gene. However, determining the precise boundaries of these regions is beyond the scope of this guide, and you need to refer to relevant literature or previous examples to figure this out. At this moment in time, we are not calling introns without experimental data.

9.4.3 Annotating wrap-around genes

Wrap-around genes are those that 'connect over the right and left ends of the phage genome. Wrap-around genes can be annotated by following the procedure above under the heading "**Annotate the Frameshift in DNA Master**", (**Section 9.4.1**). In this case, the first region will the portion of the gene at the right end of the genome, starting at your chosen start site and stopping at the end of the genome. The second region would be the portion of the gene at the left end of the genome, starting at position 1 and ending at the stop codon for the frame. For example, in a 60,000 bp genome, the two regions might be something like 58,734-60,0000; and 1-4.

There is a caveat associated with wrap-around genes. GenBank software cannot tolerate a wrap-around gene when annotated in a linear genome. Since all phage genomes are submitted as linear genomes (because in a phage, they are all linear), a gene that extends over the ends is not permitted as a CDS. However, it is tolerated if labeled as a Miscellaneous Feature instead.

9.5 Predicting tRNA and tmRNA genes

DNA Master's Auto-Annotate feature runs the tRNA search tool **Aragorn**, v1.1, which may identify some tRNA genes in your genome. However, the version of Aragorn that is within DNA Master does not call the tRNAs (and their ends) as well as it could. The newest, web-based version of Aragorn is the best of the tRNA programs at determining the correct ends of tRNAs. The other web-based program, **tRNAscan-SE**, is useful for finding non-canonical tRNAs as it is possible to relax its search parameters.

tRNAScan-SE and web-based Aragorn must be run on every sequence.

9.5.1 Running web-based Aragorn (version 1.2.36)

- Go to: http://130.235.46.10/ARAGORN/

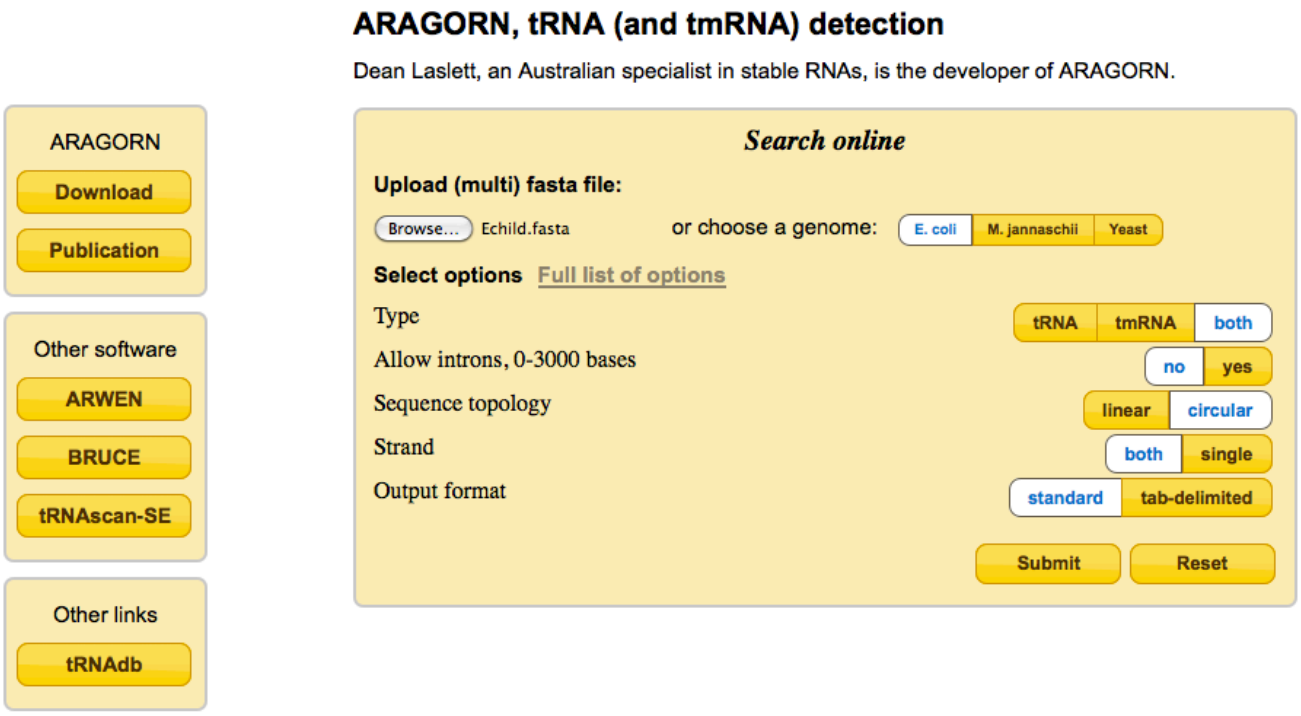


Figure 9.19

- In the '**Upload (multi) fasta file**' section, click '**Browse…**' then select your phage's DNA sequence as a FASTA file.
- Choose the following settings:

  Type: **Both** (tRNA & tmRNA)

  Allow introns: **no**

  Sequence topology: **circular** (because phage genomes circularize upon infection)

  Strands: **both**

  Output format: **standard**
- Click the '**Submit**' button.
- Your results will load in a new page. The output includes the secondary structure of the tRNAs found. An example is shown in **Figure 9.20**.

Figure 9.20

The principles underlying Aragorn are described in:

> Laslett, D. & Canback, B. (2004) ARAGORN, a program to detect tRNA genes and tmRNA genes in nucleotide sequences. *Nucleic Acids Res.* **32;**11-16. PMID: 14704338

9.5.2 Running tRNAscan-SE (version 1.23)

- Go to: http://mobyle.pasteur.fr/cgi-bin/portal.py#forms::trnascan

Click the button marked Advanced options. Scroll down through the list, and change the following settings:

Improve detection of prokaryotic tRNAs to “yes”

Analyze sequences using COVE only to “yes”

Show both primary and secondary structure components….. to “yes”

Disable pseudogene checking to “yes”

Strict or relaxed tRNAscan-SE mode to “relaxed”

Strict or relaxed EufindtRNA mode to “relaxed”

Cove cutoff score for reporting tRNAs to “0” (you have to scroll down a bit to find this one)

Save secondary structure results file to “yes”

Anything you change from the default settings will automatically get highlighted in yellow.Now if you click the "only simple options" button, all of your yellow-highlighted selected options on the advanced page should be returned to the main window, and it should look like this:

Figure 9.21

- Next to the field labeled "**Sequence file**" **, cl**ick the '**Upload…**' **tab a**nd select your phage's DNA sequence as a FASTA file.
- Now click "Run"

Note: This program can take some time to run with the relaxed parameters (~20 min), and so it may be worth pre-running it prior to class time.

When the job is finished, you will get a file emailed to you with your results that can be opened by any browser, or you can right-click on the finished job in the far left column (one is circled in the below figure) and select "Open link in new tab". This will open the results of your search.

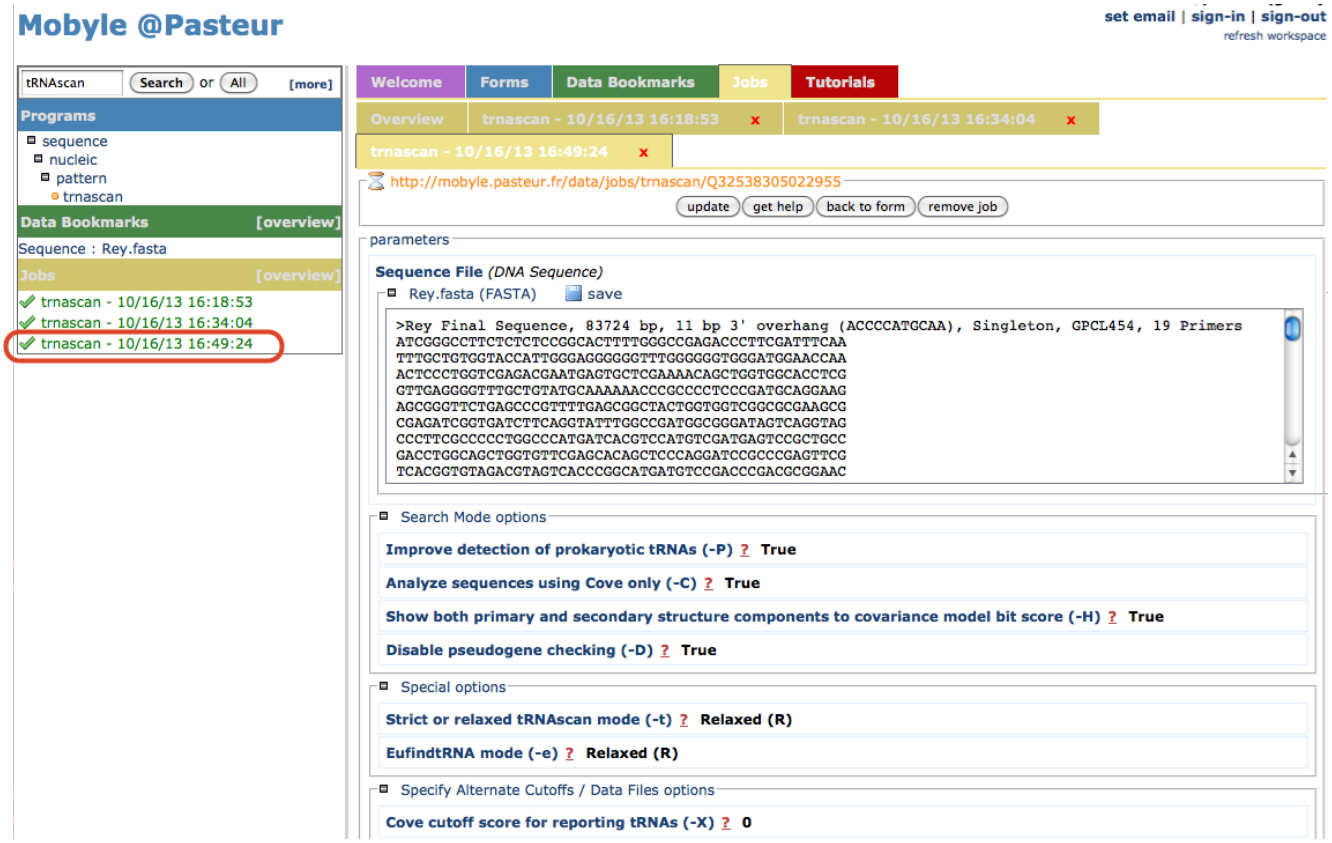


Figure 9.22

The outputs from this program looks like the sample in **Figure 9.23**

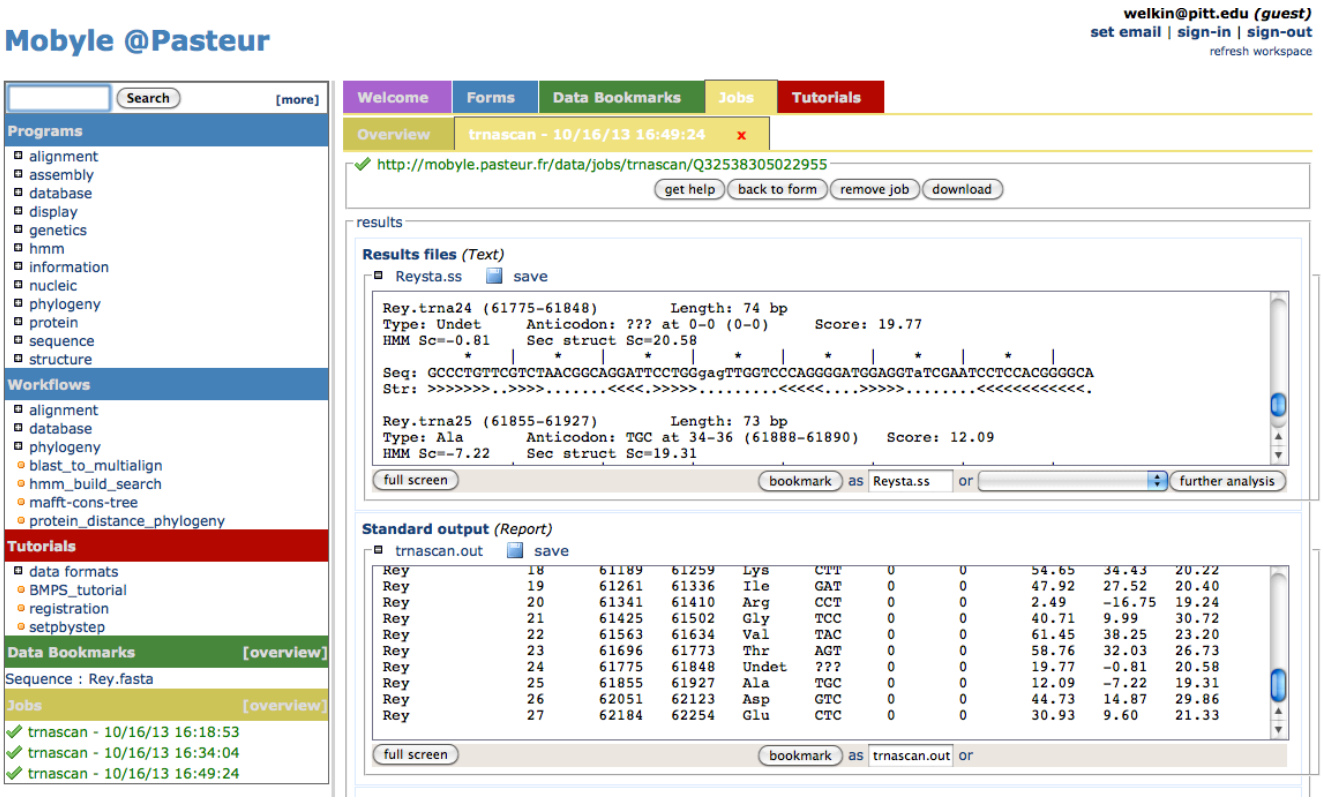


Figure 9.23

The top window displays the predicted tRNA sequence with ">>>" under the sequence to indicate portions that pair to make the tRNA stems and "…" underneath to make the tRNA loops.

The bottom window lists the number of found tRNAs, their start and stop coordinations, the amino acid, the anti-codon, the intron boundaries (almost always zeros in phages), the COVE score, the HMM score, and the secondary structure score. We are primarily interested in the COVE score as a measure of the quality of the putative tRNA.

The principles underlying the tRNAscan-SE program are described in:

> Lowe, T.M. and Eddy, S.R. (1997) tRNAscan-SE: a program for improved detection of transfer RNA genes in genomic sequence. *Nucleic Acids Res*, 25, 955-964.

9.5.3 tRNA secondary structure and end determination

Some manual checking is required to determine the precise 3′ end of a tRNA gene.

In the tRNA schematic below, the 5′ end of the tRNA is a 7 base-pair segment called the Acceptor Stem. The remainder of the tRNA is depicted in the diagram; it winds all the way through three additional stem-loops of variable lengths and then back to the matching base pairs of the acceptor stem. Conserved bases are labeled in nucleotide single-letter shorthand at the appropriate position. The tRNA algorithms score potential tRNAs based on their adherence to the conserved bases and stem-loop lengths.

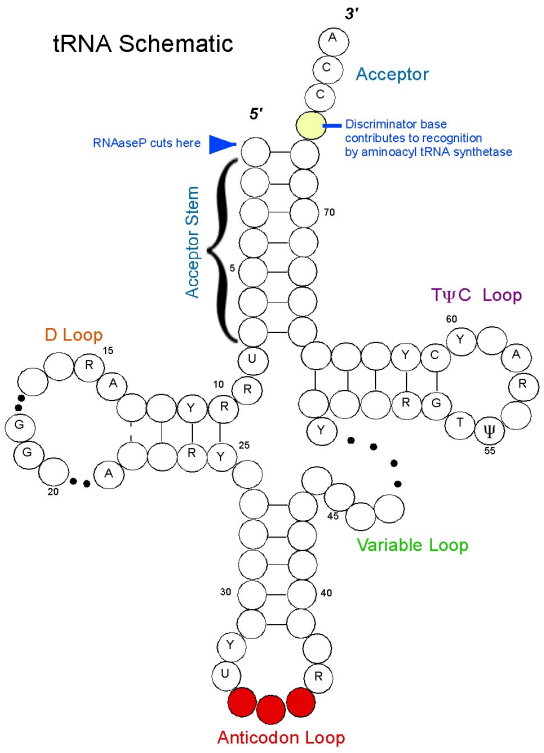


Figure 9.24

After the Acceptor Stem, the 3′ end has up to four unpaired bases. The first is called the discriminator base, and it is part of the recognition system that the tRNA synthetase uses to charge the tRNA with the correct amino acid. The discriminator base is followed by the sequence CCA.

The ends of the tRNA must be carefully checked. The acceptor stem loop must be seven base pairs. The CCA sequence at the 3′ end must be present on the final tRNA molecule for the tRNA to be charged. Sometimes in the tRNA gene within the DNA of the genome the CCA sequence is truncated, in which case the additional part of the CCA sequence is added after transcription. **Therefore if the 3′ end of the sequence is not CCA, it should be trimmed at the first deviation from the CCA sequence, and the remainder should not be included in the gene call**. **This is usually done by web-based Aragorn perfectly.**

The tRNA Schematic shown in **Figure 9.24** is an adaptation of the schematic found on the Lowe website http://lowelab.ucsc.edu/tRNAscan-SE/ with review and guidance from Dr. Craig L. Peebles.

In Summary:

The phages that contain more than 1 tRNA within their genomes tend to localize the tRNAs to certain regions of the genomes (also called "tRNA clusters" in the phage tRNA literature.) It is highly unusual that a phage will contain a sole tRNA distant genomically from all the others within its genome found by the programs, or encoded on the opposite strand as all the others, or encoded within a ORF called by GeneMark or Glimmer that has high coding potential. In general, violation of any of the three preceding conditions is sufficient for exclusion of a potential tRNA from an annotation. It is possible for a phage genome to have multiple tRNA clusters (for example, the Cluster C mycobacteriophages have three tRNA clusters).

The "best" tRNAs are those with a tRNAscan-SE COVE score higher than 20, and that are also found by web-based Aragorn. These criteria include almost all known bacterial tRNAs. Some phage tRNAs meet these standards, however, others don't. Until the phage tRNAs are more extensively tested for expression and functionality in the wet lab, we will err on the side of inclusion.

In our annotations, we will include:

- All tRNAs with COVE scores above 2
- All tRNAs found by web-based Aragorn, even if they are not found by tRNAscan-SE at all or have a COVE score lower than 2.

The ends of the tRNA should be trimmed to match the web-based Aragorn start and stop coordinates.

9.5.4 Entering a tRNA in DNA Master

DNA Master may have already called some of your tRNA genes using the old stand-alone version of Aragorn. If so, go to the **[Feature]** tab and the **[[Description]]** sub-tab, and enter the following information. (See **Figure 9.25** for an example.)

- Type: tRNA (not CDS)
- 5′ and 3′: Exact coordinates as determined above
- Feature Product: "tRNA _____" (In the blank, write the amino acid 3-letter abbreviation, e.g. "Lys".)
- Feature Notes: "tRNA _____" (In the blank, write the amino acid 3-letter abbreviation followed by the anti-codon, e.g. "Lys (ttt)".) Include the COVE score from tRNAscan-SE and whether or not it was detected by Aragorn.

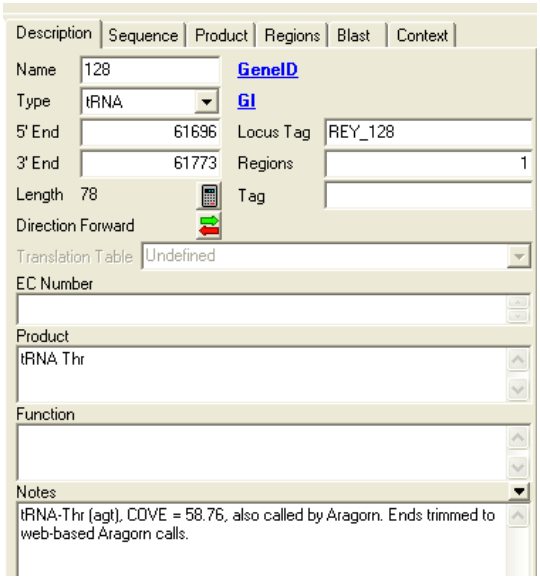


Figure 9.25

If you are adding a brand new tRNA, click the '**Insert**' button at the bottom of the central column. Then enter in the above information in the window that opens and click '**Add Feature**'. (You can leave the name blank, and it will be automatically assigned when you renumber genes, as described in **Section 9.3.3**.)

9.5.5 Identifying and annotating tmRNA genes

Description from Wikipedia:

"Transfer-messenger RNA **(tmRNA)** is a bacterial RNA molecule with dual tRNA-like and messenger RNA-like properties. In *trans*-translation, tmRNA and its associated proteins bind to bacterial ribosomes which have stalled in the middle of protein biosynthesis, for example when reaching the end of a messenger RNA which has lost its stop codon. tmRNA can recycle the stalled ribosome, add a proteolysis-inducing tag to the unfinished polypeptide, and facilitate the degradation of the aberrant messenger RNA."

The coordinates for tmRNAs can be annotated as web-based Aragorn (or the algorithm BRUCE on the Aragorn web page) calls them. Entering tmRNAs into your DNA Master annotation can be done using the same procedure as for entering tRNAs (**Section 9.5.4**), only the "**Type**" of feature in the should then be "tmRNA" (not CDS or tRNA).

9.6 Documenting your gene calls

Just like in at the wet bench, it is important to takes notes and document your findings during genome annotation. While you may want to keep an additional notebook or word document for lengthier rationales or questions, there is a good place to put an abbreviated version of your rationale for each gene in the DNA Master file. In the **[Feature]** tab and **[[Description]]** sub-tab, there is a convenient box marked "Notes" that will allow you to do this.

Every gene call should be documented in its Notes as described below. These notes are extremely important for the annotation review process. This is the place where you will want to advocate for those difficult calls. Once checked, these notes will be removed from the GenBank submission file.

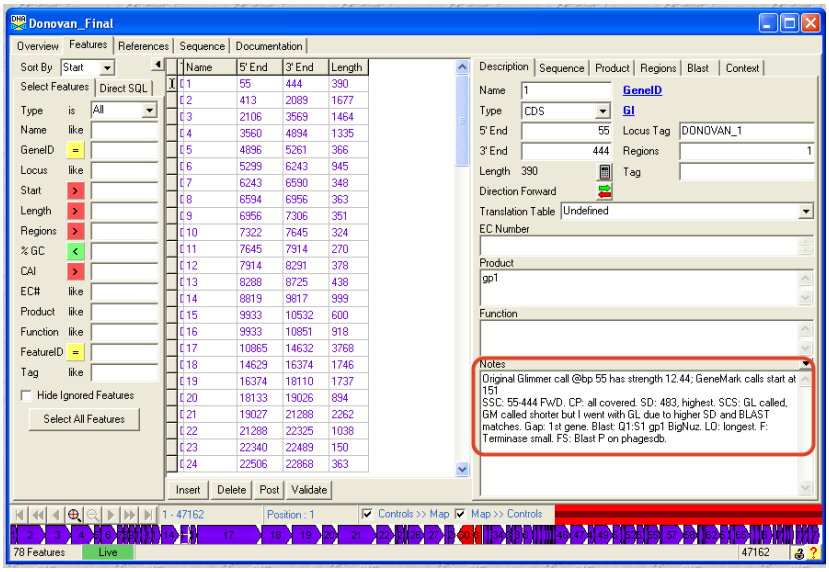


Figure 9.26

To edit the Notes field, simply click within the field and type. Make sure you Post changes (**Section 9.3.1**) when done so that you don't lose your work. The following information should be recorded for every gene, in order if possible.

- The original auto-annotation information. So do not delete!
- **SSC:** Start/stop coordinates. (This may seem redundant because there are "Start" and "Stop" fields that already contain this information, but it serves as a double-check that all changes you made are actually contained in the final file.)
- **CP:** Whether or not your start includes all the coding potential identified by GeneMark.
- **SD:** Whether or not the start has the best SD score of all this ORF's possible starts.
- **SCS** (Start choice source): Whether or not the gene was called by Glimmer and GeneMark, and if the start was called by same.
- **Gap** (or overlap): Any significant gap or overlap with preceding gene (in basepairs).
- **Blast:** The best BLAST match, and the alignment of the gene start with that BLAST match. (For example, "Matches KBG gp32, Query 1 to Subject 1", or "Aligns with Thibault gp45 q3:s45".)
- **LO** (Longest ORF): Whether or not the coordinates you have chosen yield the longest reasonable possible gene for that ORF. A start that overlaps the upstream stop codon by 4bp can reasonably be called the "longest ORF" if the only other start choice would cause a 700bp overlap with the preceding gene.
- **ST:** Starterator data is entered here. If it is not applicable, record NA. If it was run and was not informative, write NI. Other notations are "suggested start" or "conserved start" (a start found throughout the analyzed pham that isn't the suggested start).
- **F** (Function): Gene Function
- **FS** (Function source): source for the function (see **Section 10**), and supporting evidence (BLAST e value? HHPred probability? Phamerator?). Please mention if BLASTP assignments come from phagesdb.org or from GenBank. *Include the phage name and gene number of the phage used as the basis for your functional assignment*. Only enter the putative function in the Notes, **do not write anything into the function field.**
- Anything else you think is important. In particular if you made a different choice than previous annotators have made in published genomes, and feel very strongly about your choice, this is the place to let us know. **Example:** If your gene start does not match the published starts of similar genes in GenBank, an explanation of why not. ("Published Thibault gp45 start not present in my sequence" or "Thibault start caused a 200 bp overlap with upstream gene")

An example of good Notes:

SSC: Start: 2435 Stop: 2650 (FWD). **CP:** Agrees with both Glimmer and GeneMark predictions. **SD**: 310, best score. **SCS**: ORF includes all coding potential shown on GeneMark-Smeg output. **LO:** 213 bp; longest possible ORF. **Gap:** 84 bp gap with Previous Gene. **BLAST:** gp3 of Oline; Oline aa 1 aligns with query aa 1. **ST:** Not informative; **F** (Function): NKF (No Known Function).

10 Assigning gene functions

10.1 Overview

Before the age of bioinformatics, the only way to determine a gene function was to perform wet bench experiments: cloning and expressing a gene, or knocking a gene out, and then characterizing the resulting mutants. These kinds of studies are still the gold standard for determining gene function.

Because of recent advances in sequencing technology, however, we are identifying potential genes far more rapidly than we can perform the supporting wet bench experiments for functional determination. Bioinformatic tools can make some strong predictions through comparative approaches, especially by comparing the sequence of any particular gene to the sequences of genes with known functions (i.e., those that have been characterized experimentally).

Even with the new tools that are available, we are unable to assign functions to the majority of the genes that we annotate in bacteriophage genomes. A common occurrence is that students assign poorly-supported gene functions. All reference to functions need strong evidence to be considered.

There are several categories in which genes can be assigned functions with some confidence.

1. **Virion structural and assembly genes**, i.e. those encoding proteins that are either components of virion particles or assist in their formation. These include genes encoding the terminase, portal, capsid maturation protease, scaffolding proteins, major capsid protein, major tail subunit, tail assembly chaperones, tape measure protein, and minor tail proteins.
2. **Genes involved in phage DNA replication**. These include DNA polymerase, DNA primase, DNA helicase, nucleotide metabolism genes, and ssDNA binding proteins.
3. **Genes involved in life cycle regulation**. These include various regulators such as repressors and activators, integrases, recombination directionality factors, etc.
4. **Genes involved in lysis**, including endolysins (referred to as Lysin A in the mycobacteriophages), Lysin B, and Holins.
5. **Other well-characterized genes**, including transcription factors, toxin/anti-toxin systems, peptidases, phosphatases, host gene homologues, methylases, nucleases, and DNA binding proteins, among others.

Not all phages contain all of the above genes—or at least genes that can be recognized as having these functions (e.g., we still are not sure where the tail assembly chaperones are in the cluster B phages). Even with a substantial body of knowledge about the mycobacteriophages, we can still only assign functions to 10-20% of the genes in a given genome. Remember that it is okay to write "No Known Function" or "NKF" for a gene.

For more information on the specific function of some of the above phage genes as they relate to mycobacteriophages, see:

http://phagesdb.org/glossary/

Remember assigning functions is an area where the amount of data that is available for comparison is greater than ever before. There is a lot of history to how you can attribute function to the gene product you are investigating. As with everything else you have learned in the annotation process, this requires that you contextualize the evidence you are evaluating. In addition, we are getting better at this, so comparisons to more recent annotations is a better reflection of our understanding than older annotations. If it looks too good to be true, it probably isn't! You can always write a note that there was a weak 'hit' to some obscure function, but DO NOT CALL gene function without good evidence.

10.2 Using bioinformatic tools to assign gene function

There are four main tools that are useful for predicting potential gene functions. These are:

1. BLASTp (at PhagesDB or NCBI)
2. HHpred
3. Phamerator
4. Conserved Domain Identification (either through NCBI or Phamerator)

10.2.1 BLASTP

BLASTP [BLAST (Basic Local Alignment Search Tool) P (Protein)] is a program that searches your query protein sequence against all known predicted protein sequences. You have already come across this in the context of using BLAST to refine your annotations, but it is very useful for predicting potential gene functions.

There are three basic ways of doing BLASTP searches. They can be done:

1. within the DNA Master environment (**Sections 4.5 and 9.3.4**)
2. on phagesdb.org **(Figure 10.1)**
3. at BLASTP on the NCBI BLAST server. (**Figure 10.2**)
   http://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastp&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome

When you BLASTP your protein sequence, you are comparing it to all the other protein sequences in your database. If you are using BLASTP through DNA Master or on NCBI's website, your database is GenBank. So even though the results are derived from the same the same database, they display is different. This BLASTP is important to do because this is where your protein sequence is compared to the latest information across all of the protein sequences in NCBI's non-redundant protein databases. This is where you can find 'new' information.

If you are using BLASTP on PhagesDB, your database is comprised solely of mycobacteriophages QC'd by the University of Pittsburgh or mycobacterial proteins. This is valuable because it gives you information that we know about. Remember when you use this that you will see lots of DRAFT annotations. You will want to pay attention to the QC'd entries (those whose name does not contain the word "Draft").

Some Pointers:

- BLASTP at PhagesDB is fast! Actually, it is very fast!
- BLASTP at NCBI and PhagesDB is pointing to information that was input by people, so human error exists. In addition, some folks use an automated process without review, so the perpetuation of those errors is rampant. At all times, you will need to evaluate the data you accumulate.

When assigning functions using BLASTP you should consider the following points.

E value. E values are a measure of the likelihood that this alignment would appear at random. Therefore, lower E values are better (less likely to be random) matches. For any potential functional match, the E value should be 10^{-7} or less. The E value should be evaluated in the context of the length of the alignment. (See next point!)

The length of the alignment. Does the alignment extend the entire length of your protein? If it only matches a portion, you should interpret this cautiously. For example, if you find a relatively small segment of a protein that matches others at a statistically significant level, you may want to consider annotating this as a domain rather than a full protein function. For example, if a small segment of your protein matches other proteases, you might want to consider writing "peptidase domain", rather than "peptidase" in your Notes.

Likelihood of the proposed match. Even if you have an exact match to a piece of a protein in *Vinis vinifera*, it is pretty unlikely that a protein from grapes has the same sequence and function as a protein in a mycobacteriophage. Most of the time when BLASTP aligns bacteriophage proteins with eukaryotic proteins, the alignment is occurring between repetitive sequences, rather than the functional domains of the protein.

Figure 10.1 is an example of a good BLASTP match, generated using NCBI's web-based BLASTP, where a putative function can be assigned. In the graphical portion of the results, there are many matches in red (the color for the highest match scores) that extend over the entire length of our query sequence. In the list of matches, we can see that all of the E values are well below 10^{-4}. Many of the hits have a Description that involves terminases. We can now say, with some confidence, that the protein we BLASTed is a terminase.

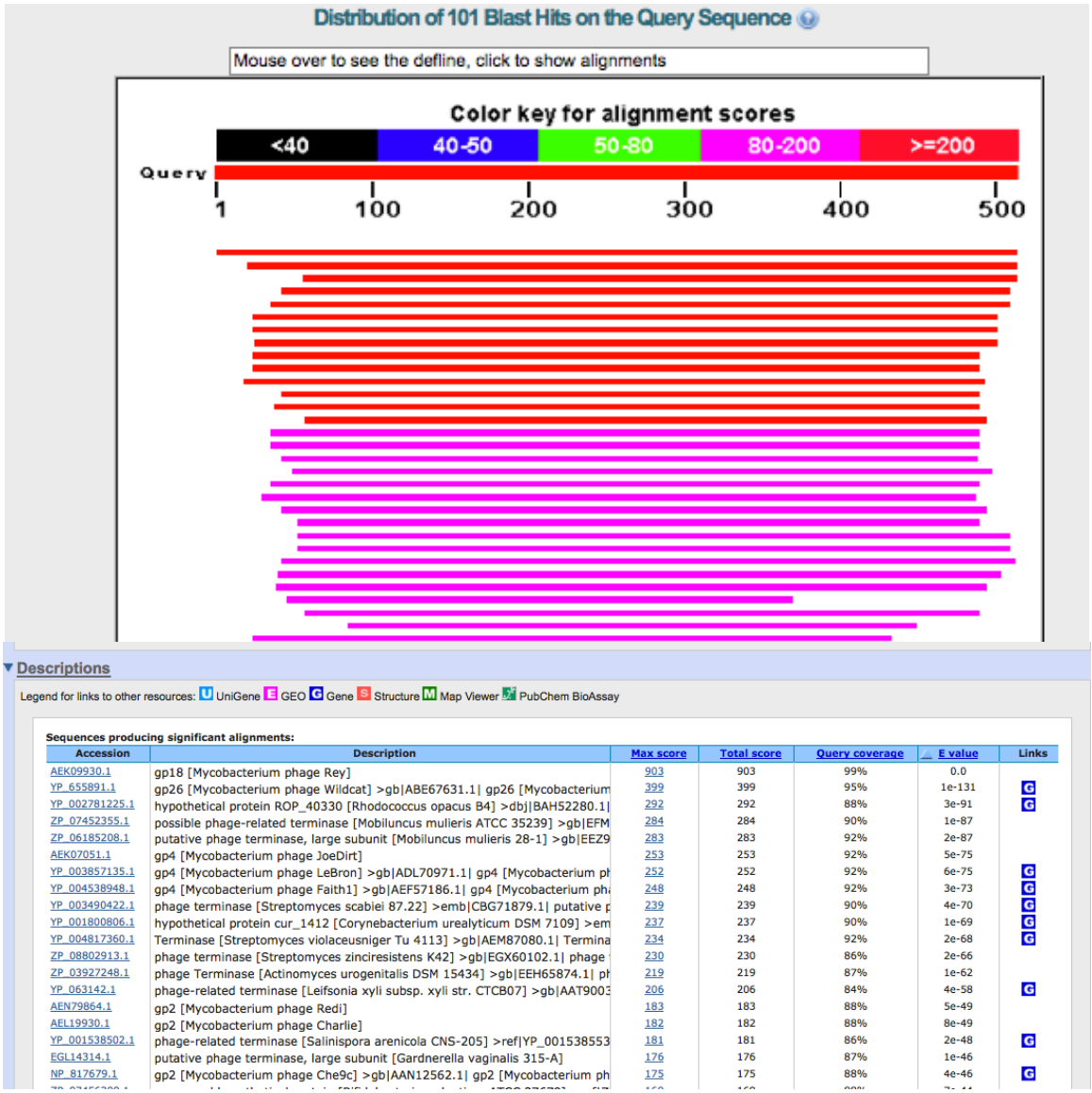


Figure 10.1

BLASTing a protein on phagesdb.org:

From the top banner, choose BLAST, and select BLASTP from the dropdown menu. Paste your protein sequence in fasta format into the search window, and select the database you would like to search against. The remainder of the settings are similar or identical to the setting choices you see with the program on the NCBI website.

Other than the database, the primary difference between searching GenBank and searching the PhagesDB is in how the results are displayed. We have included both the gene number and the function on the result on PhagesDB to aid functional assignments and highlight mosaicism of the genomes. Gene number and functional assignment are in two different fields in GenBank files and the BLASTP output on NCBI only returns what is written in the product field in a GenBank file.

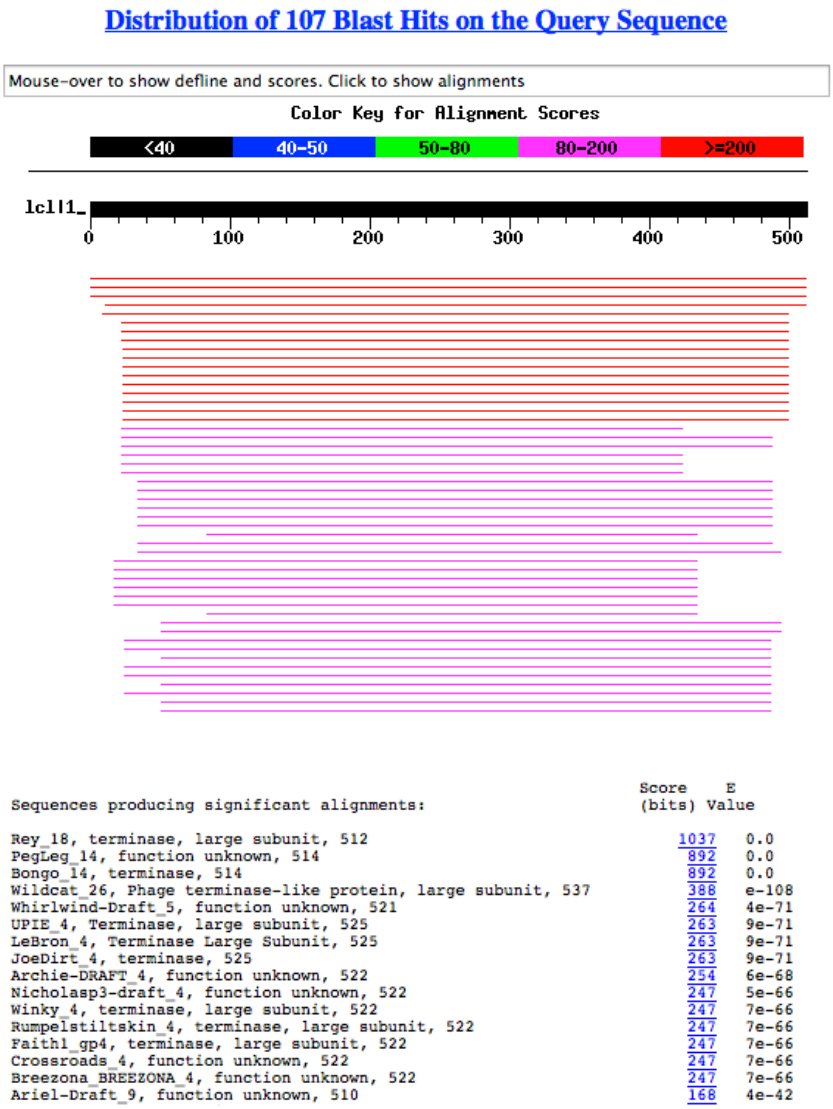


Figure 10.2

10.2.2 Conserved Domain Database (CDD)

When you run your protein sequence through BLASTP on the NCBI webpage, one of the default settings is to examine your protein sequence for conserved domains. Conserved domains are smaller shorter amino acid sequences that are usually affiliated with a specific part (or domain) of a protein. These conserved domains also appear on Phamerator maps as yellow boxes *within* a gene's colored box if they have been enabled through the View menu on the map. You will want to evaluate a CDD match just like you do a BLASTP: look at the E value, the length of the alignment, the likelihood, AND the usefulness of the CDD assignment.

If you have a conserved domain detected within your protein, the function assigned to the domain will be frequently—but not always—be similar to ones found in BLASTP matches. Useful domains to indicate in your annotation are things like peptidases or phosphoesterases, but there is a wide variety that may appear.

Not all conserved domains will be useful. Some contain little information, such as "Conserved domain of unknown function, found in bacteriophages". Others are false positives such as the "Structural maintenance of chromosomes" domain that often appears in structural proteins.

Unfortunately, it is not clear *a priori* which are false fits and which are reliable. Consideration of the genomic context as well as the HHpred search described below are perhaps the most reliable indicators.

An example of a reliable Conserved Domain hit reported by BLASTP on the NCBI server might look like: If you hover your cursor over these boxes with the mouse, a pop-up window will appear that tells you about the conserved domain. When you click on the pink Terminase_1 box in the provided example, you would detect an E value of $2e10^{-15}$.

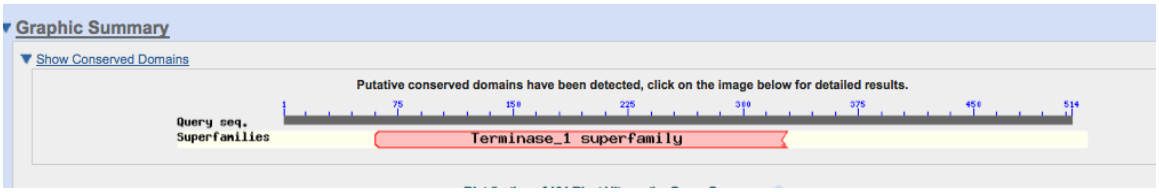


Figure 10.3

The same gene in a Phamerator map might look like:

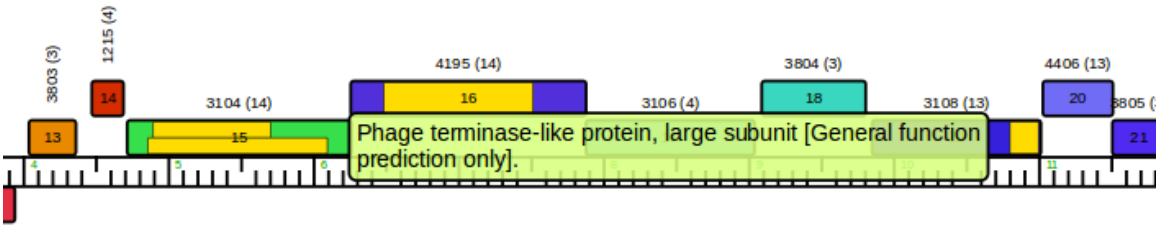


Figure 10.4

In this case, we moused over gene ***15*** in **Figure 10.4**, and the green box describing the domain appeared. A less informative match on NCBI might look like:

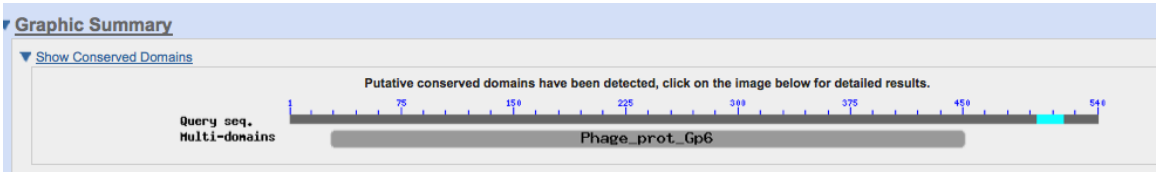


Figure 10.5

We already know that this is a phage protein, so this is not particularly useful information. And the same gene in Phamerator:

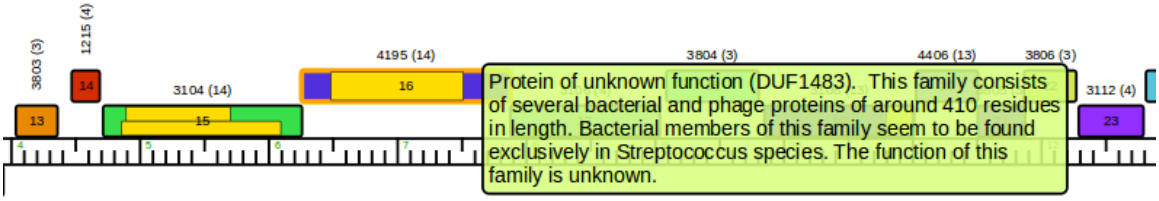


Figure 10.6

In this case, we moused over gene ***16*** in the above map, which is the well-characterized portal protein (shown in BLASTP hits). Based on the notes in the green box, we see that the Conserved Domain Database does not know that this is the portal protein. This is an example of the dependence of GenBank on its authors, who may not be as informed as they should be.

10.2.3 HHpred

HHpred is essentially a more sensitive way of searching for functions than BLASTP. In detail:

> HHpred performs an iterated multiple sequence alignment using your query amino acid sequence and its best GenBank matches, using either PSI-BLAST or HHblits (Homology detection by iterative HMM-HMM comparison). It then builds a Hidden Markov Model (HMM) based on the alignment, and compares this model to HMMs based on the Protein DataBank (PDB) (which contains crystal structure coordinates for crystallized proteins). By comparing conserved residues to a 3-D coordinate map, we can sometimes detect and assign gene functions to genes that have very few informative matches using BLAST.
>
> For more information about the design, abilities, and bioinformatics of HHpred, see:
>
> http://toolkit.tuebingen.mpg.de/hhpred/help_ov

Like BLAST, some matches in HHpred are very useful while others are more likely to be false positives.

To run HHPred, go to http://toolkit.tuebingen.mpg.de/hhpred.

You can register on this site, but it is not necessary. (Anonymous job results are stored **two weeks**, whereas jobs of logged-in users are stored for at least **two months.)** We recommend that you store this data in some fashion (it should save completely in html format. The interpretation that is written in the notes may not be enough information to prove a case to assign a function.

To run a protein sequence at HHPred refer to **Figure 10.7**:

- Copy your sequence and paste it in the appropriate window (A & B)
- Choose 3 databases available in the list (they can be simultaneously used): pdb70_[currentdate], PfamA_[currentdate],, and tgrfam_[currentdate], (C.) See http://toolkit.tuebingen.mpg.de/hhpred/help_params#databases for more information.
    - Pdb70 is the Protein Data Base that contains all publicly available 3D crystal structures of proteins.
    - PfamA is a database of curated alignments of genetically mobile domains found in signaling, extracellular and chromatin-associated proteins
    - TGRFAMs is a collection of curated protein families that provides a tool for identifying functionally related proteins based on sequence homology.
- Name the job. We suggest phage name_gene number OR phage name_STOP coordinate (D.)
- Click the Submit button (E.)
- Check the status of the job by its color. (F.)

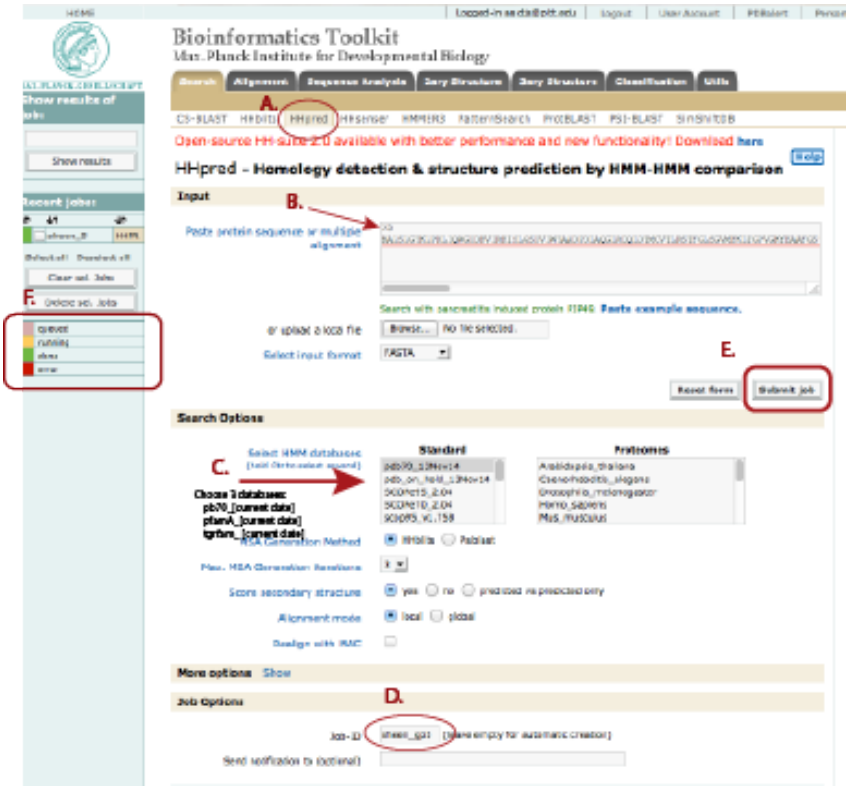


Figure 10.7

The results can look like **Figure 10.9**. Note the following

- Green here indicates the job is finished. (A.)
- Each bar represents an alignment that is linked to more information below. It follows the rainbow color scheme, with red showing the highest probabilities (95- 100 %). This demonstrates the length of the alignment. (B.)
- Each line in (B.) has a probability (C.) and a definition (D.).
- Once you get to where the description and alignment are (E.), you can evaluate if the structure has the same function in your phage genome. Remember functions have to be appropriate for phages!

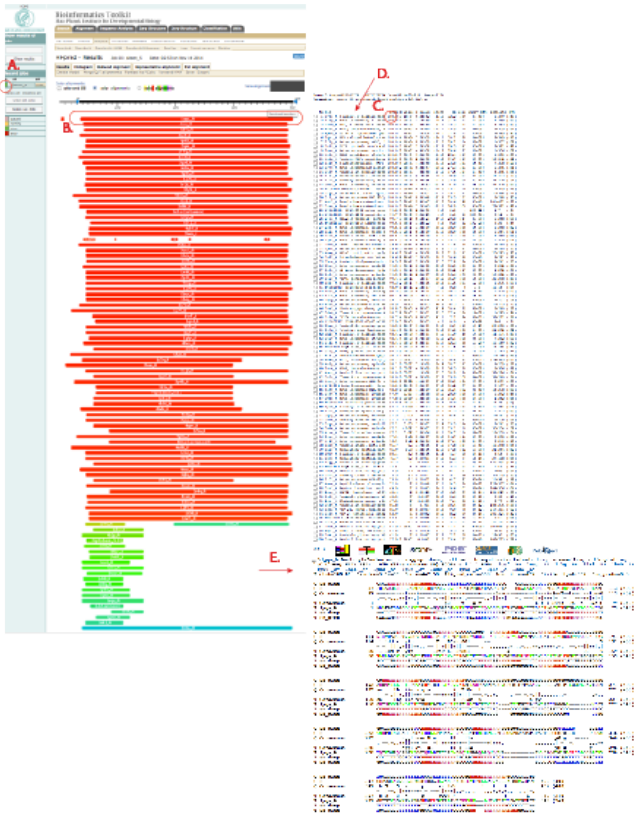


Figure 10.8

Figure 10.8: (This representation has been compressed.)

Like BLAST, HHpred provides a graphical view where the best matches are shown in red and lower-quality matches go to orange -> yellow -> green -> blue -> black. Also like BLAST, below the graphical representation is a list with useful information, including the score each hit gets. In the above screenshot, the best hit, "`D-alanyl-D-alanine carboxypeptidase`" (this is the PDB designator, if you want to see the crystal structure), matches your protein with 100 % probability and an E value of $3.1e^{-57}$.

Good HHpred matches have high probabilities (90 or above), and low E values (the lower the better). The scientists who wrote HHpred claim that matches with probabilities above 30% might be real matches. However, if you are going to claim a function found in HHpred with a probability between 30 and 90%, supporting data (such as a conservation of a domain, a function found in other mycobacteriophages, or synteny) is necessary.

For more on determining if your HHpred hit is a real match, see:

http://toolkit.tuebingen.mpg.de/hhpred/help_faq#correct%20match

When we scroll down to the look at the specifics of the alignments, we see:

Another example of an informative report from HHpred is below.

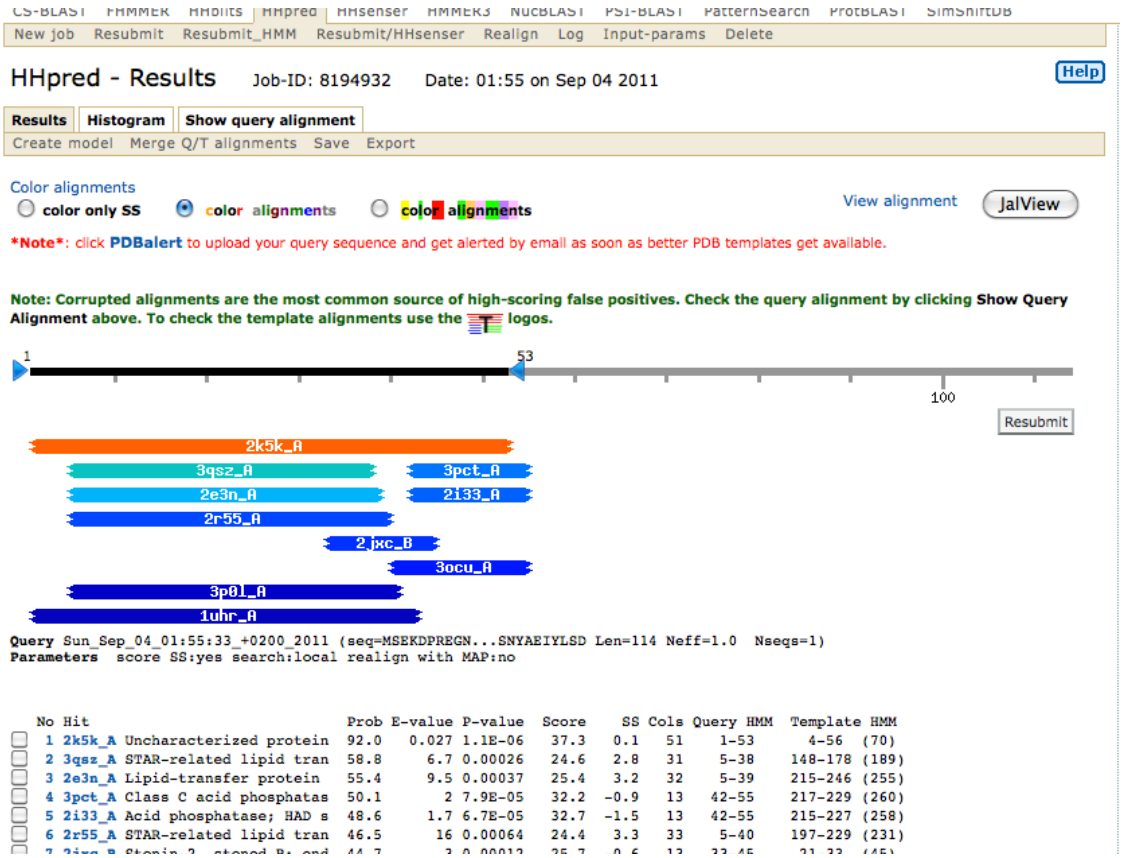


Figure 10.9

The top hit, to 2k5k_A, has a Probability score over 90, and it is an uncharacterized protein. The rest of the matches have low probabilities (80 or below), and high E values. So even though the other matches are to phosphatases, and one might be tempted to write "phosphatase", this would not be a supportable functional prediction for this protein.

10.3 Other ways to assign gene function

10.3.1 Synteny

Many of the genes in bacteriophage genomes—but especially in the virion structure and assembly genes—appear in the same order (synteny). Therefore, sometimes functions can be inferred from gene order. The typical order is:

Terminase → Portal → Capsid Maturation Protease → Scaffolding → Major Capsid Subunit →

Major Tail Subunit → Tail Assembly Chaperones → Tape measure → Minor Tail Proteins

Sometimes other smaller genes of unknown functions are interspersed within the structural genes, but in general the overall order remains conserved. While we may see conservation of gene order in some other areas of phage genomes, these other areas are far more mosaic than the structural genes are, and so the use of a synteny argument applies primarily when assigning gene function to the virion structure and assembly genes.

The longest gene in the genome of a phage with a flexible tail is almost always the tape measure protein gene. This gene is directly proportional to the length of the tail in the flexible-tailed phages.

10.3.2 Prior functional assignments

Many of the genes within the previously sequenced mycobacteriophages have already been assigned functions based on experiments, BLAST and/or HHpred matches, or synteny. Do not assume that all functions are known and recorded in our database. There is new data available all the time and should be reviewed. Even Dr. Hatfull periodically reviews the mycobacteriophage genomes and updates gene functions to genomes that were annotated long ago. If you are trying to assign a function to a gene that has a BLAST match to or is in the same pham as one of the genes with an assigned function in our published literature, you may assign your gene the same function.

10.3.3 Phamerator

Many of the genomes in Phamerator have already been published according to the most recent functional assignments, but not all. We are constantly in the process of improving our gene calls, and so Phamerator functional assignments reflect our best effort at assigning gene functions **at the time the QC'd form of the genome was entered** into Phamerator. This means that many of the more recent genomes might have better functional assignments than some of the older ones. If you're using comparisons in Phamerator to already-published genomes to determine function, your best source of gene function are available in the Phamerator Map, with **Descriptions** enabled through the View menu of the most recently published Mycobacteriophage genomes. **These same Phamerator descriptions also appear in the results line in PhagesDB BLASTP searches.**

11 Merging and checking annotations

11.1 Merging overview

In a classroom setting, different portions of a genome are often assigned to different students or groups of students to annotate. Once all portions have been annotated, they must be combined into a single file, and the "**Merge**" function in DNA Master performs this action. It takes multiple files from a single phage genome and creates a single master file that contains all of the gene calls from each individual file.

Note: merging will **only work on files that contain identical sequences**. If you are going to split a genome among different annotation groups, make sure that you keep the entire sequence intact, and simply work on a region identified by gene coordinates (e.g. between 20,000 and 30,000).

Typically, you'll merge all of a given genome's partial annotations together into a single file that can then be proofed and edited to become the final complete annotation. However, it is also possible to do several iterations of merging. For example, if two groups are working on the region from 10,000 to 20,000, you may want to merge their files first, come to a consensus on that region, then merge the newly checked version with the other final files from other sections of the genome. Merging is flexible enough to meet your pedagogical goals.

11.2 Merging multiple annotations into a single file

- Collect the files you'd like to merge into a single directory. Remember that these must all be from an identical DNA sequence (i.e., the same phage genome).
- You may want to include a newly made auto-annotated only genome. Go back to phagesDB.org to obtain the FASTA file and quickly auto-annotate. Use this as your reference file in the merged data. If the sequence has been corrupted in any of the student files, the merge will not work. This is an excellent quality control measure!
- Open DNA Master.
- Go to **File → Merge**

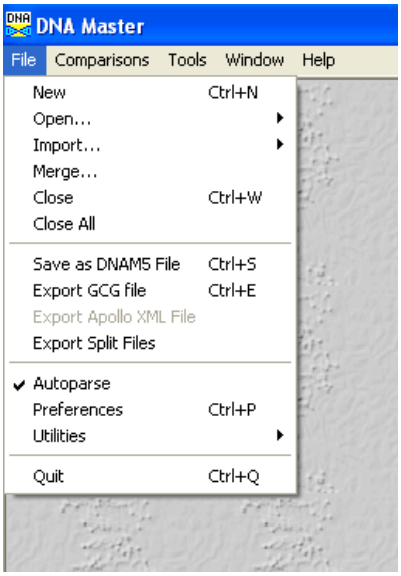


Figure 11.1

- A new window will open, as shown below.

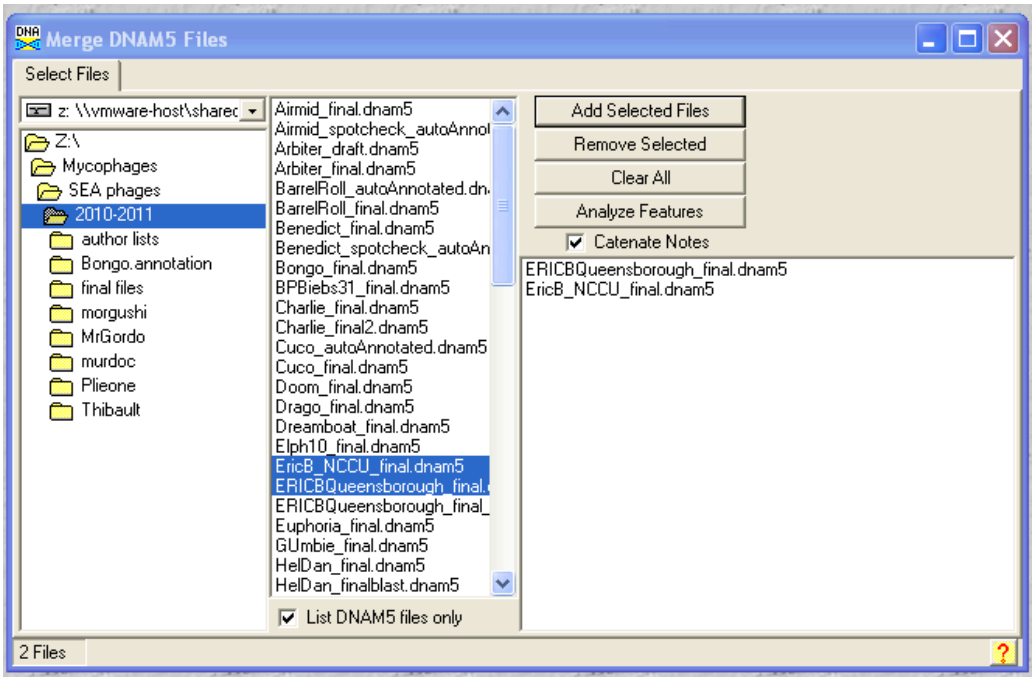


Figure 11.2

- In the left column, browse to the directory on your computer that contains the DNA Master (.dnam5) files that you want to merge.
- In the center column, click on files that you want to add to your merged file.
- Click the '**Add Selected Files**' button. The files will then appear in the empty white box on the right. You can browse to additional directories (if necessary) to add additional files.

- Once all of the files that you would like to merge added, they will be listed in the white, check the box marked "**Catenate Notes**".
- Click the '**Analyze Features**' button.
- The window will open a new tab, **[Merge Files]**.

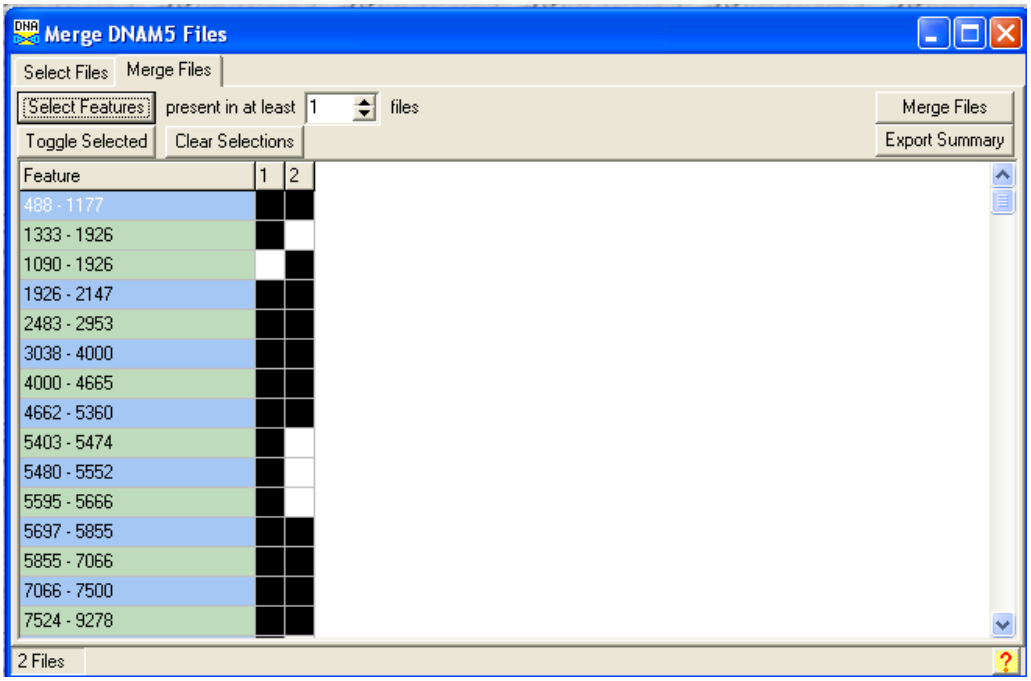


Figure 11.3

In the picture above, Features (or gene calls) are listed according to genome coordinates. Each file you selected is represented by a numbered column, displayed in the order that they were selected in the previous tab.

In each row, a black box is present if that file contains that feature, and a white box is present if the file does not contain that feature. The first feature, 488-1177, is present in both of the files that were merged. The next feature, from 1333-1926, was present only in the first file. The third feature, from 1090-1926, was present only in the second file. Because both of these features have the same stop codon, what we are looking at is a disagreement in the two files about where the start for this gene should be. File 1 calls it at 1333, while file 2 calls it at 1090.

- To export a spreadsheet that contains the above information (which can be useful to identify areas of disagreement that require further attention), click the '**Export Summary**' button in the top right of this window.

To create a .dnam5 file with all of the gene calls from the files to be merged:

- Click the '**Select Features**' button. (Selected features will turn red, as shown below.)

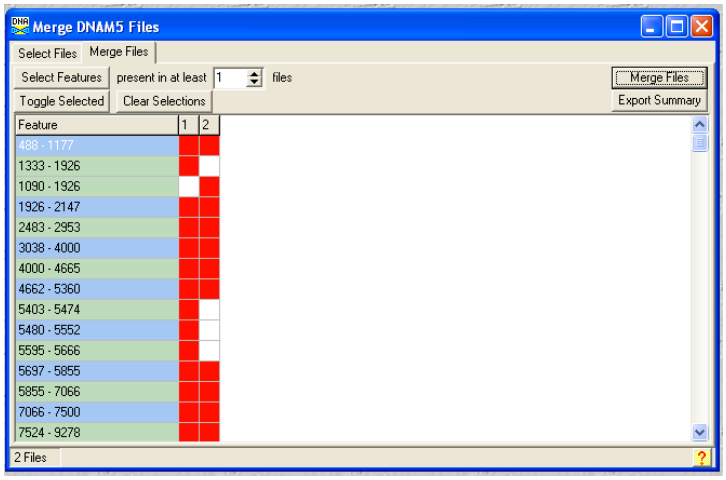


Figure 11.4

- You can tailor your selection by modifying the number in the dropdown box next to "present in at least ___ files". After changing the number, click the '**Clear Selections**' button to erase previously selected genes, then click the '**Select Features**' button again to make your new selection. In the picture below, now only the features present in at least two (both) files are selected and shown in red.

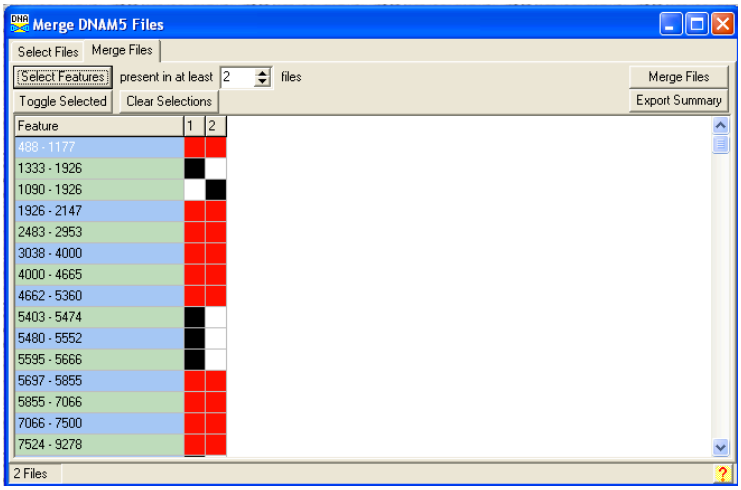


Figure 11.5

- Once you have selected the features you would like in your merged file (picking all of them is a good choice, disagreeing features can always be deleted from the merged file after review), click the '**Merge Files**' button at the upper right corner.
- A new window titled '**Merged Sequence**' will appear, as shown below.

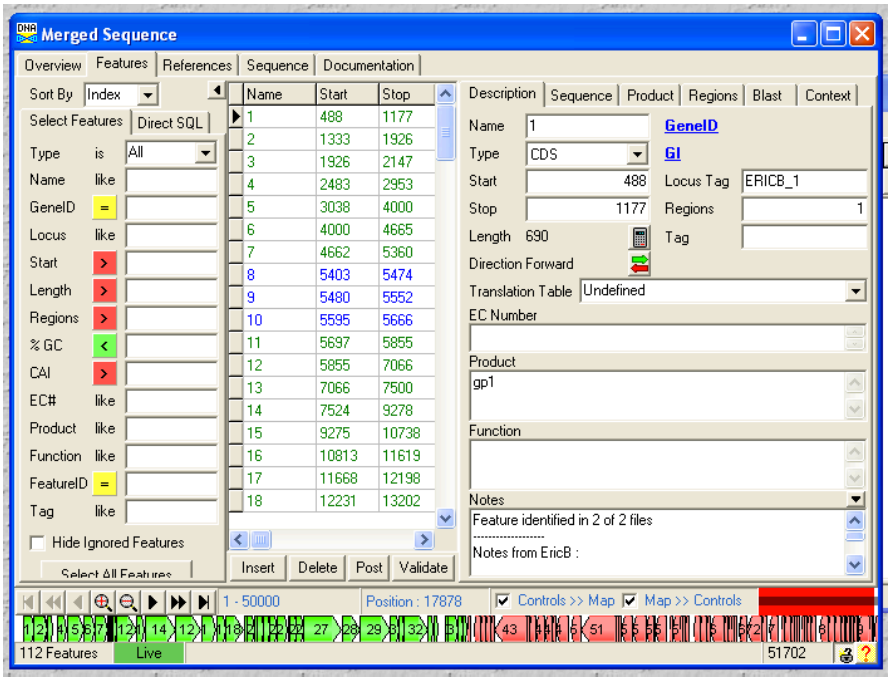


Figure 11.6

- Save your file immediately by going to: **File → Save as DNAM5 File**
- Select a meaningful name for the merged file, such as "YourPhageName_Merged.dnam5".

In the above picture, we are looking at feature 1. In the "Notes" field on the lower right, the top line indicates that this feature was called in 2 of 2 files. Further down in the Notes box, both sets of notes have been concatenated.

How features and notes are reconciled when there is disagreement:

While all the genes from the unmerged files will be present within the features of the merged file, DNA Master will not treat all these genes equally. Features that share the same stop codon but have different start codons will be listed as separate features in the merged feature list. Features that were selected by the majority of the files in the merge will be given preference in the merged file, and will be listed first in the feature table if it is sorted by Index.

The most popular features will have concatenated notes. That is, all the notes from the unmerged files will be listed in the Notes field of the merged feature. Less popular features will be in the merged file, but will be listed at the end of the feature list when sorted by Index. Less popular features will have their original notes, not merged notes.

- To clearly see discrepant calls, go to the "Sort By" drop-down menu at the top left of the **[Feature]** tab, and select "Start" rather than "Index".

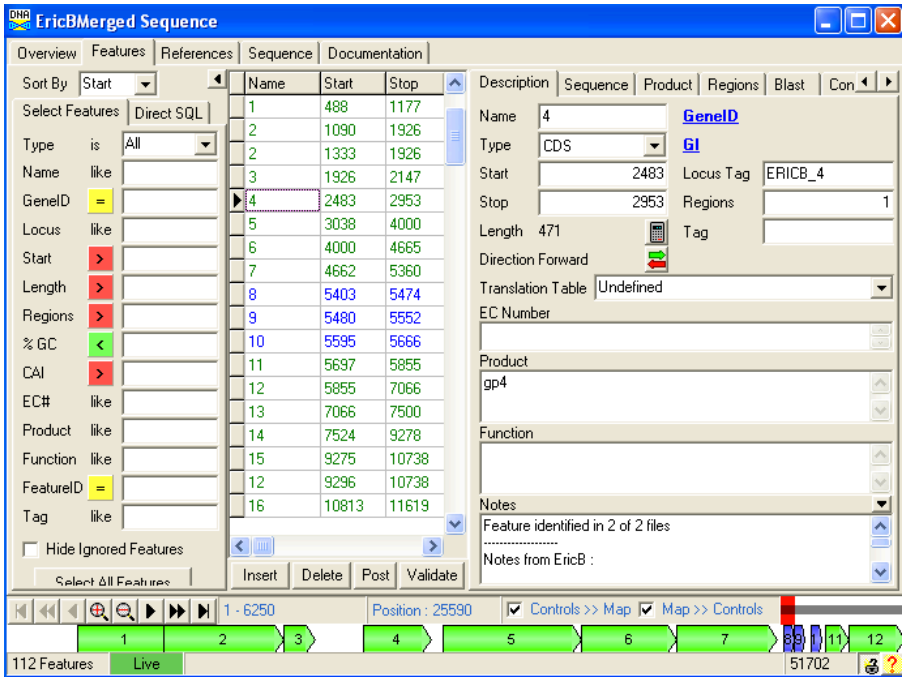


Figure 11.7

You can see that there are two versions of gene 2, one from each file, that share the same stop codon but differ in their choice of start codon. Now it's up to you to determine which is correct!

Note: if students are only working on a portion of the genome, it may be advantageous to delete the auto-annotation calls from the portions of the genome they are not working on prior to merging. That way, only the reviewed features will be in the merged file instead of many unreviewed computer-called features. Remember, only delete the features you don't want to merge, not the underlying sequence.

11.3 Checking an annotation

Once you've merged all files, made final decisions on each gene, and believe you've finished your annotation, there are a few final steps to take before submitting your genome for review and then GenBank submission. The steps below reflect what we typically do at the University of Pittsburgh to quality-control submitted annotations, so you can stay one step ahead and try to identify any remaining issues first.

- Review your cover sheet and check that you have correctly formatted and re-Blasted your phage DNA Master file. Annotations will be returned if all components are not completed.
- Click the '**Validate**' button bottom of the central column in the **[Feature]** tab. The response should be "All ORFs appear valid." If you get a different message here, check the gene(s) identified for errors.
- Zoom in on the interactive map along the bottom of the sequence, and carefully scroll along the whole length of the genome. Do all the genes seem to be tightly packed? Look for large overlaps, gaps, or duplications. You can also do this by generating an ORF Map (**Section 5.2**)

- Open an interactive Phamerator map of your phage along with two or three closely related cluster members that are already in GenBank. (Remember that it is still your auto-annotated genome in Phamerator.) Are there any areas where your phage has orphams (white boxes) or otherwise diverges from similar phages that you have **not** addressed during your refinement? Or you can address these same questions using The Genome Comparison Tool in DNA Master. See Exploring Bacteriophage Biology at Protocols at phagesDB for instructions.
- Re-BLAST your genome. The BLAST data must match the final calls of your phage genome final file. Don't guess or presume that the Blast data is up-to-date. ReBLAST for final review.
- Create a "Genome profile". This is a spreadsheet (.csv format) of all the information in the Features table. While this won't give you any new information compared to simply scrolling through your features, it may help you make sure you don't miss anything or that you have recorded in formation in an incorrect field.

  Go to: **Genome → Profile**

  In the window that opens, there are a number of settings. The default settings should be fine, but consider checking the "Export Notes" box if you'd like Notes included in your spreadsheet, and consider unchecking the "Load into Excel" box if you don't have Excel or would like to open the file later. You may also export "Product", "Function", and "Best BLAST hit" for your final review. You can quickly evaluate if your file is in tip-top shape.

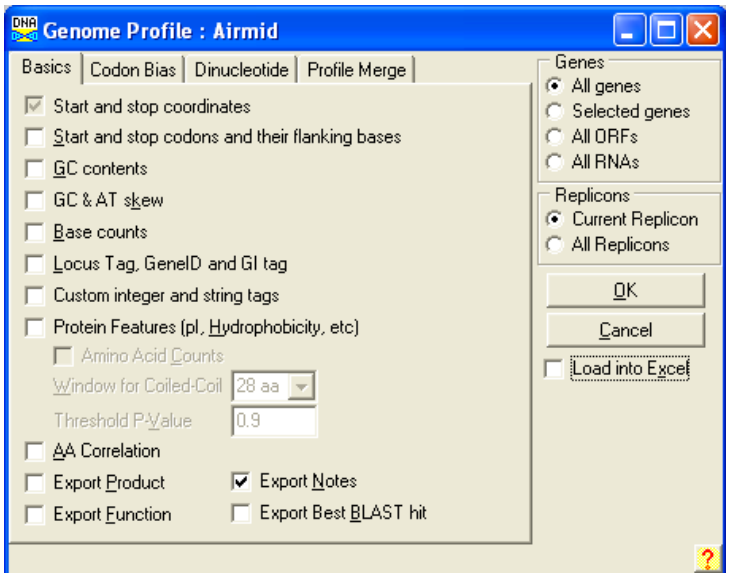


Figure 11.8

- Now check each gene individually.

  Read the comments, and consider: Do the start and stop coordinates listed match the coordinates in the file? Does the gene have Glimmer/GeneMark support? A good RBS/Shine-Dalgarno score? Include all the GeneMark-Smeg coding potential? Is the gene as long as possible without overlapping the previous gene too much? Match its best BLAST hit 1-to-1? If the phage has close relatives in GenBank (you can tell pretty quickly by using Phamerator), our frequent default position is to make a newly annotated gene match the

annotated genes already in GenBank. If it doesn't, use your best judgment based on the other metrics.

Check the gene functions, and consider: Do they make sense? Are reported E values low (below 10^{-7})? Do they match the Hatfull-approved data (where appropriate)? Is there a source listed for a function (HHpred, BLASTP, CDD, GFHmap, other)? If there is no known function, is "NKF" written?

When checking tRNAs, consider: Is the tRNA amino acid and anti-codon written in the Notes box? Does the tRNA end with "CCA", and if not is it trimmed correctly? Did you report the COVE score and if it is found by Aragorn?

For gaps in your gene calls, consider: Is there an ORF with coding potential that was missed? Are there any BLASTX hits with good GenBank matches?

Keep track of any potential issues you encounter during checking, and revisit those areas of the genome to ensure the best call has been made. Make a note of all issues, and include them in a cover sheet to be submitted with your final annotation.

12 Submitting final files for review and GenBank submission

You've made it. Plowed through gene after gene, pored over BLAST results and coding potential diagrams, perhaps argued over some start sites, and have merged all calls and come up with a final annotation.. Congratulations!

But you are not quite done. Science is always in the details and there are details to be done!

To submit your competed project for QC, you still need to:

- Make a duplicate copy of your file, erase all of the notes, and add functions into the notes field. This is merely a formatting issue, but will help in the QC process.
- Upload both versions to PhagesDB
- Upload an author list to PhagesDB
- Upload a cover letter to PhagesDB

The submit annotation button is located on PhagesDB under the Data button.

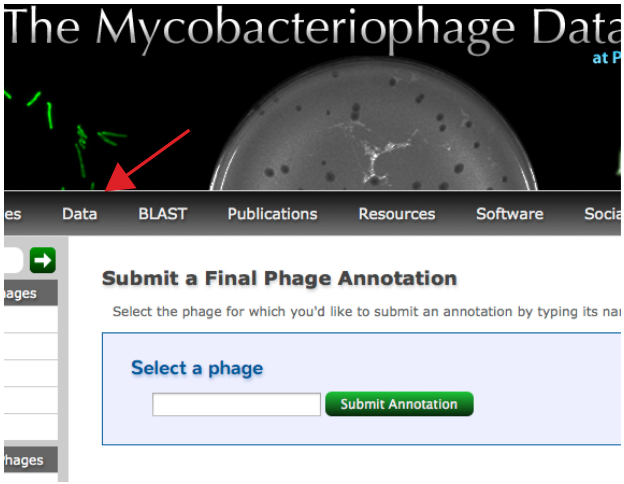


Figure 12.1

After expert review, your annotation will be either accepted or returned. If accepted we will provide a GenBank flat file for your inspection. Once accepted, the file is then sent to GenBank. If not accepted, your file will be returned with an explanation and request for revisions.

12.1 Details of your final DNA Master (.dnam5) file

A final .dnam5 file is one that has the following properties.

1. It must be named "YourPhageName_CompleteNotes.dnam5", which will help distinguish it from other versions you may have been working on.

2. **Do not delete the original Auto-Annotation remarks.**

3. **It must contain one entry and set of notes (and only one) per feature**. That means that if you have merged multiple files, you need to have evaluated the data from each source, come to a decision, and deleted erroneous versions of each feature. There should also be only **one set of notes** for each feature, and it should contain

everything listed in **Section 9.6** about proper documentation of your gene calls. You may have to delete some notes, or even rewrite some notes from scratch to meet this criterion.

4. All features must be validated (**Section 9.3.2**).
5. All features must be re-numbered if necessary **(Section 9.3.3**).
6. Recreate the Documentation (**Section 1.4**).
7. All features must be re-BLASTed (**Section 9.3.4**).
8. Any functions are noted in the Notes fields, along with their source. (**Section 9.3.3.)**
9. The correct format will look like **Figure 12.2**.

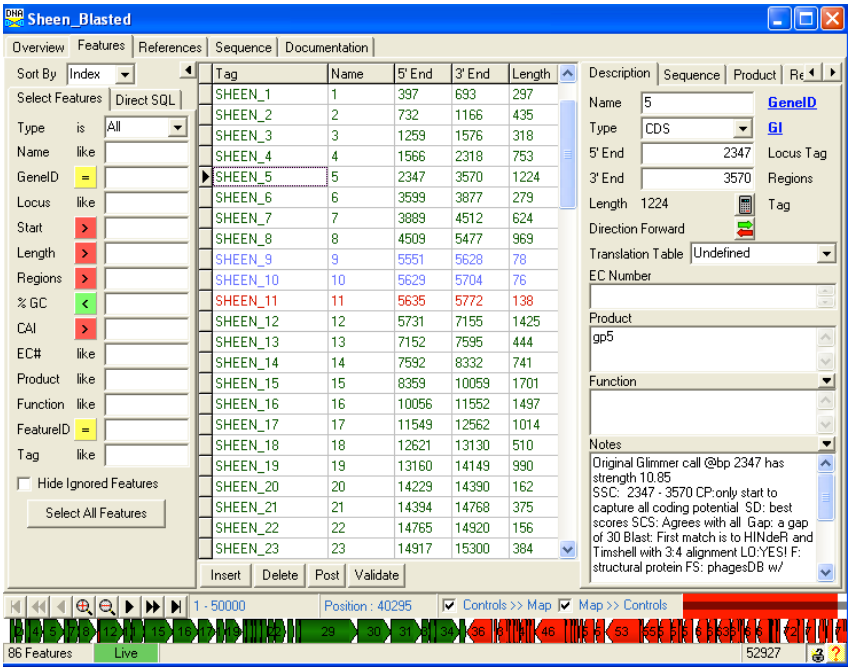


Figure 12.2

12.2 The second "minimalistic" Final .dnam5 file

This file will contain only the formatting for GenBank submission. Now that you have arrived at you're your best annotation, we are ready to present it as complete and polished. To do so, we remove all notes except REPORTABLE functions. This year, with the increase in numbers of genomes, we are asking that you do this step. This step may also help you to separate out the functions you wish you could call and the ones that you are willing to stand behind and truly defend. There are a couple of ways to do this, but here is our advice for a simple approach:

- Create a Profile of your genome (**Section 11.3**). This will put your features in an Excel spreadsheet.
- Erase all notes (**Figure 12.3**).

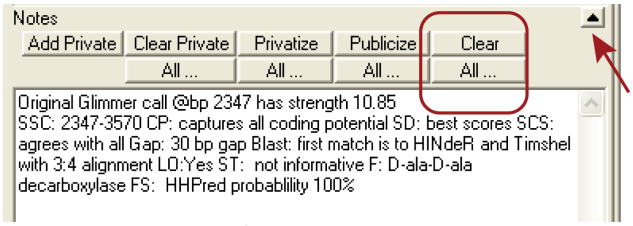


Figure 12.3

- Add reportable functions into the empty notes fields, using the profile as your guide (**Figure 12.4**).

Figure 12.4

- Recreate documentation (**Section 1.4**).
- Save file as "YourPhageName_Final.dnam5".

12.3 Details of your author list

Please create a list (.csv formatted file) of the authors from your school who are to be included in this GenBank submission. Your author list should meet the following criteria.

- It contains **ONLY** authors from your school who deserve to be listed on the GenBank file. **Do not** include names from Pitt, HHMI, sequencing centers, or any other source.
- It is a .csv file. A .csv formatted file can be created in Excel, using the '**Save as…**' function, and selecting .csv as the file type.
- It contains exactly three columns, with **NO HEADERS** at the top of each column.
- The first column contains the last name, the second column contains first name, and the third column contains a middle initial. **If no middle initial is needed, type a period in that column instead. All three columns should contain some information for each author.** See below for an example.

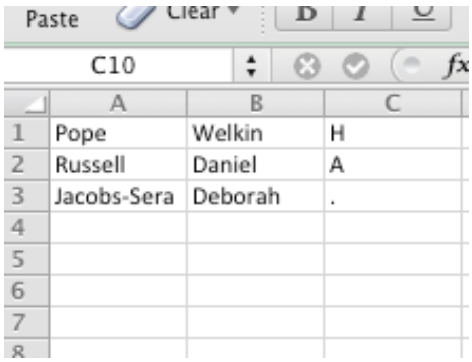


Figure 12.5

12.4 Details of your cover sheet

For submission along with your annotation, **please create a document with a brief list of genes in the genome that you feel warrant extra attention by the annotation quality control team prior to submission and why**, including but not limited to: start choice, functional assignment, or gene inclusion/exclusion; and/or areas that you have extensively investigated and you feel should remain genome gaps. Do not send this information in the text of the email, but rather as an attached document. This list should not be more than a page. If you feel confident in all areas of your annotation, please state so. Upload it along with your 2 .dnam5 files when complete.

Acknowledgements

DNA Master was designed and developed by Dr. Jeffrey G. Lawrence at the University of Pittsburgh. The program has gone through a multitude of advances, some of which were implemented by Dr. Adam Retchless when he was a graduate student with Jeffrey. Dr. Lawrence continues to provide support, updates and new functionalities to DNA Master.

DNA Master is much more than a genome annotation tool, although this is its main role in this guide. DNA Master has been developed for assisting in bioinformatic dissection of genomes – primarily microbial – with a view to understanding how they have evolved and how they are related. As you become familiar with the program and develop your own interests in genome evolution, we hope these utilities will be of use to you.

We are deeply grateful to Dr. Lawrence for making DNA Master available to us and for his constant willingness to listen to our suggestions and our particular needs. Over many years we have found DNA Master to be an incredibly effective platform for genome annotation and analysis, and Jeffrey's contributions cannot be overestimated.

We would also like to thank the literally hundreds of students and faculty who have used DNA Master and provided feedback that has helped us to develop and refine this annotation platform.

We thank our colleagues in the Science Education Program at HHMI, especially David Asai, Kevin Bradley, Lucia Barker, Razi Khaja, and Melvina Lewis, for their tremendous insights and feedback. Melvina F. Lewis provided the terrific cover design.

The electronic version of the guide is available on the wiki and PhagesDB. Additional helpful documents at PhagesDB include:

- **System requirements and Installation of DNA Master**
- **DNA Master Quick Start Guide**
- **Gene Function with Bench Support and References**
- **Case Study: The Annotation of Etude**
- **Exploring Bacteriophage Biology**