Investigating promoters in O cluster phages

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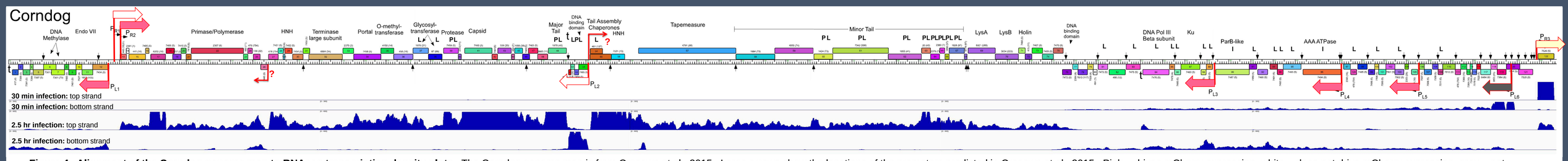
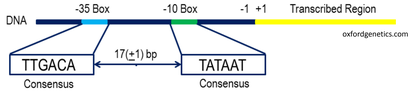


Figure 4. Alignment of the Corndog genome map to RNAseq transcription density plots. The Corndog genome map is from Cresawn et al., 2015. Large arrows show the locations of the promoters predicted in Cresawn et al., 2015. Pink = drives mCherry expression, white = does not drive mCherry expression, gray = not cloned yet. Small arrows are proposed promoters in this study (see Fig. 3). RNAseq transcription data is displayed in four tracks: the two top tracks indicate RNA quantities isolated early in infection (30 min) – forward strand above and reverse strand below. The bottom two tracks indicate RNAs isolated late in infection (2.5 hr) – forward strand above and reverse strand below. Height of blue histogram indicates quantity of RNA detected from that position in the genome. All tracks are shown at same scale.

INTRODUCTION

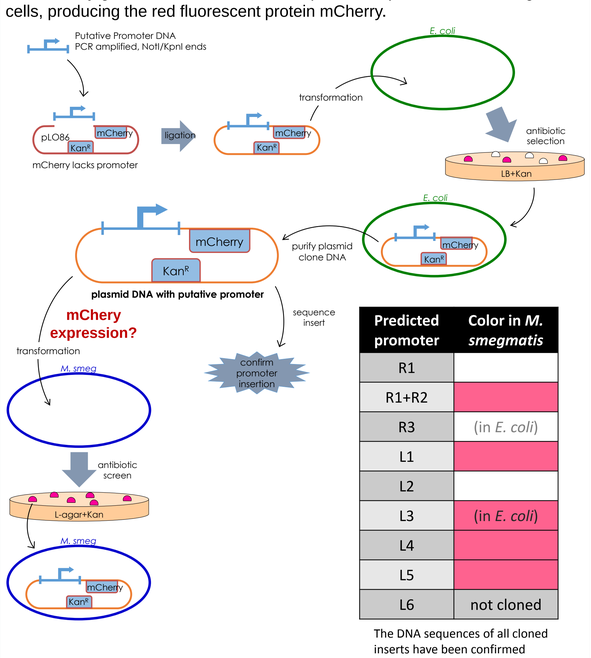
Promoters are DNA sequence elements that serve as binding sites for RNA polymerases to initiate transcription. Some promoters in mycobacteriophage genomes are bound by the sigma factor SigA within RNA polymerase (Hatfull, 2014). These promoters can often be predicted bioinformatically by searching for sequences that SigA (or its *E. coli* ortholog Sigma70) bind to. Such promoters have two regions of conserved consensus sequence, the -35 region and the -10 region.



We are interested in how gene expression in phage genomes is regulated. As a first step, we are studying the promoters of the genomes of O cluster phages. The locations of nine SigA-like promoters were predicted in these genomes (Cresawn et al., 2015; see Fig.4).

Previous work

To test the activity of the predicted promoters, candidate DNA fragments were cloned into an mCherry expression plasmid (Oldfield and Hatfull, 2014). If a DNA fragment contains an active promoter, then transcription and translation of the mCherry gene should occur when the plasmid is placed into *M. smegmatis* cells, producing the red fluorescent protein mCherry.



Predicted promoter	Color in *M. smegmatis*
R1	
R1+R2	
R3	(in *E. coli*)
L1	
L2	
L3	(in *E. coli*)
L4	
L5	
L6	not cloned

The DNA sequences of all cloned inserts have been confirmed

Research Questions

- Within the ~200 bp clones, are the predicted sequences actually required for transcription?
- Will promoters retain activity when integrated into the bacterial chromosome?
- Can additional, non-predicted promoters be cloned and studied?
- Can the mCherry expression be quantified?

RESULTS

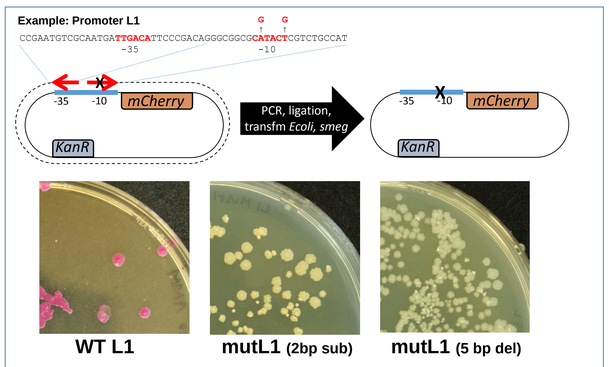


Figure 1. The predicted promoter sequences L1, L4, and L5 are necessary for transcriptional activity. The Q5 Site-directed mutagenesis kit (NEB) was used to alter 2-3 bp in the predicted -10 region (based on Oldfield and Hatfull, 2014), and to delete 5 bp between the -10 and -35 regions. DNA sequencing confirmed the mutations. When promoters L1, L4, and L5 were mutagenized, mCherry expression was absent, indicating that the promoter structure was compromised by the mutation. The other promoter clones with activity (R2, L3) have not been tested yet.

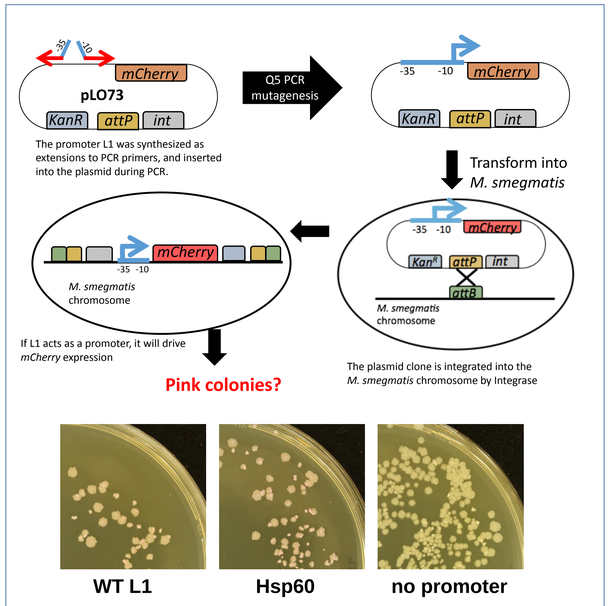


Figure 2. Promoter L1 drives mCherry expression when integrated into the *M. smegmatis* chromosome. The L1 promoter was cloned into the mCherry integration plasmid pLO73 using the Q5 Mutagenesis kit (NEB). 5' tails carrying the promoter sequence were synthesized. PCR and ligation generates circles with promoter insertions. Clones were confirmed by DNA sequencing, then transformed into *M. smegmatis*. KanR transformants are expected to carry the plasmid integrated at the Tweety attP site.

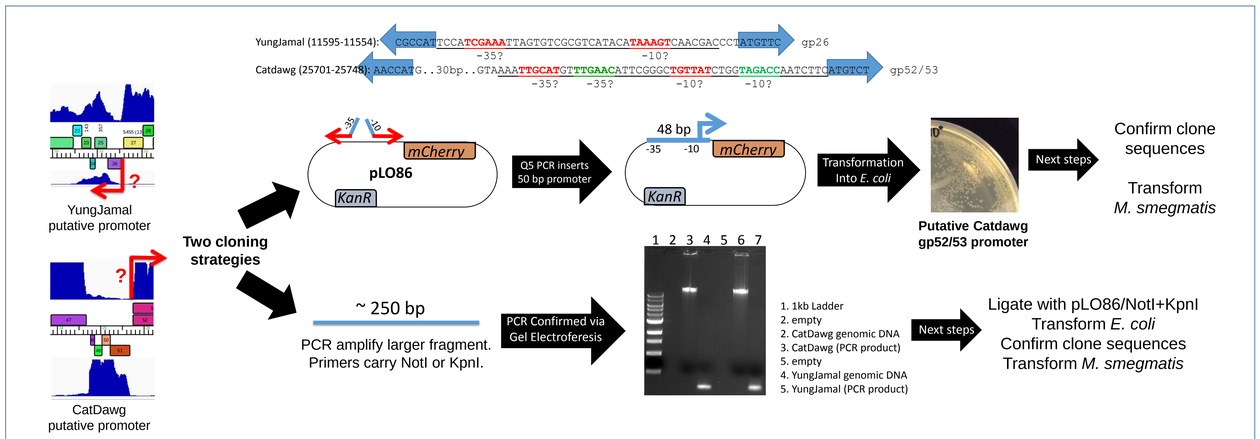


Figure 3. Two promoters predicted by genome structure and RNA expression may not drive mCherry expression when only 48 bp is cloned. We expect that promoters may exist in parts of genomes where genes point in different directions, such as at ~12,000 bp and ~26,000 bp. To examine levels of transcription in these regions, and across the genome, RNA was isolated from *M. smegmatis* cultures infected with Corndog after 30 min and 2.5 hr and sequenced by Illumina sequencing (RNAseq, see Figure 4.) Two regions of O cluster genomes were identified as potential locations for promoters driving transcription of gene 26 and the tail assembly chaperone genes. 48 bp upstream of YungJamal gene 26 and CatDawg genes 52/53 were cloned into pLO86 (identical to Corndog genes 26 and 54/55, respectively). The DNA seq of the clones needs confirmation. The cloning of 250 bp from these regions was initiated, but not completed yet.

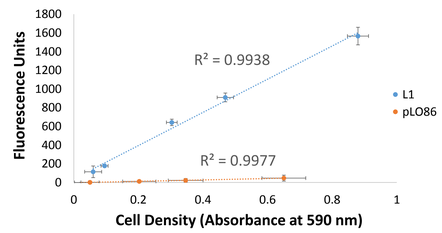


Figure 5. *M. smegmatis* clones expressing mCherry emit red fluorescence highly correlated to cell density. We wish to measure mCherry expression by quantitively measuring red fluorescence. In this way, we will be able to compare different promoter clones to each other for strength of transcription. *M. smegmatis* clones were grown to high density over 4 days, then serially diluted. 200 ul was placed in the wells of a flat-bottom 96 well plate and read with a BioTek Synergy HXT. For cell density, absorbance at 590 nm was measured. For mCherry fluorescence, samples were excited by 580-590 nm and emitted light of 612-628 nm was measured. These conditions will be used to measure all the clones produced in this project at cell densities under 1 A_{590}.

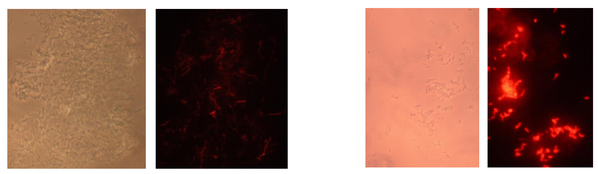


Figure 6. *M. smegmatis* carrying pLO86 clones emit a small amount of red fluorescence. We consistently measured more fluorescence in pLO86 clones than in *M.smeg* with no plasmid. To find out whether these clones were fluorescing, we examined them on a fluorescent microscope. We do not know whether this is autofluorescece or mCherry from a low level of basal transcription activity from the plasmid.

CONCLUSIONS

1. **Computational predictions were correct for promoters L1, L4, and L5.** Why do some promoters not drive mCherry expression? L2 is upstream of genes that are highly transcribed during infection. Perhaps an activator protein (phage or host encoded) is only present during infection? Or an activator binding site was not cloned into the plasmid?
2. **Q5 site-directed mutagenesis kit efficiently mutates cloned promoter, or inserts new promoter.**
3. **Promoter L1 still exhibits activity when integrated into the host chromosome.** mCherry expression appears to be lower than in the episomal plasmid. We do not know whether this is due to the plasmid copy number differences, or repression upon integration. O phages are not temperate, so the promoter doesn't need to be repressed during lysogeny.
4. **Two predicted promoters do not appear to drive mCherry expression as 48 bp clones.** The structure of the clones needs to be examined and put into *M. smegmatis* to test activity. Larger fragments will also be tested.
5. **We found conditions to measure mCherry expression quantitatively.**

LITERATURE CITED

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