Developing a Non-toxigenic Reporter Strain of *Clostridium botulinum* to Monitor Toxin Gene Induction

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**Abstract**

For purposes of detection and decontamination, existing surrogates for spore-forming organisms are unable to adequately recapitulate the relevant properties of the potent botulinum neurotoxin (BoNT) and its producing organism. Because of this, the development of surrogates for *Clostridium botulinum* is highly desirable. This effort is directed towards the creation of a line of non-toxigenic surrogate strains that produce fluorescent reporters of toxin expression, and can be used as markers of spread of both the organism and the toxin molecules. These strains will be exempt from CDC Select Agent regulations due to wholesale deletion of the protease domain of the toxin, which will be replaced by a fluorescent protein fusion.

**Background**

Many bacteria are known to cause disease in humans, but there are few that produce as deadly an effect as *Clostridium botulinum*, due to the neurotoxins (BoNT) that are produced. Studies by the CDC have indicated a relatively low incidence of naturally occurring outbreaks of botulism, making a large outbreak indicative of a potential bioterrorism event. Terrorists have attempted to use BoNT in the past as a biowar; both Iran and the former Soviet Union had large production facilities and the Japanese cult Aum Shinrikyo dispersed aerosols at multiple locations in downtown Tokyo.

Historically, genetic manipulations in Clostridia have proved difficult due to the unique requirements for growth in strict anaerobic conditions and the lack of effective genetic tools. However, using a newly developed genetic techniques for modifying Clostridia, the protease domain of BoNT is being replaced with a fluorescent protein reporter by homologous recombination [1]. Here, the requirement of oxygen for the formation of the fluorophore for this reporter system is circumvented by using the flavin mononucleotide binding fluorescent protein (FlpF) gene [EvGlow-Clostridia, Evotecat, GmbH; [3]]. Understanding both the positive and negative attributes of botulinum toxin, as well as the formation of the toxin complex (Figure 2), will provide insight for developing medical countermeasures as well as identifying targets, predicting exposure dose variations, and informing battlefield risk assessment.

![Figure 1: Pictorial Representation of Clostridium botulinum with evoglow\(^*\) replacing botulinum toxin](image)

**Methods**

As depicted in Figure 3, evoglow\(^*\) flanked by "750bp of chromosomal Clostridium botulinum DNA (corresponding to the 5’ and 3’ regions of the BoNT/A gene) was produced by amplifying each segment of DNA by PCR and combining them using the standard Gibson Assembly method. This gene fragment was then cloned into pLRRT, containing the genes for origin of plasmid transfer, origin of plasmid replication, as well as chloramphenicol resistance, the sequence specific mRNA endonuclease and its corresponding transcriptional regulator.

The resulting plasmid, containing an anaerobic green fluorescent protein (green box in Figure 4), is introduced into the recipient *Clostridium botulinum* strain by conjugal transfer. Homologous recombination (HR) of the plasmid into the desired locus is directed by using a new system developed by al-Hinai et al. [2] with designed flanking regions to the selected botulinum toxin locus (yellow in Figure 4), resulting in a chloramphenicol resistant strain.

Recombinants are selected and screened by PCR to insure integration has occurred in the expected orientation. Upon confirmation of successful integration, the second round of homologous recombination is induced by the inactivation of the mazF gene on the plasmid whose expression is toxic to *Clostridium*. The resulting strains should lack the integrated plasmid and retain either the parental or replaced allele. We will confirm the absence of the BoNT gene in the resulting strains by Taqman PCR (arrows in Figure 4) and whole-genome sequencing.

![Figure 3: Plasmid Construction](image)

**Current Progress**

- Anaerobic growth of Clostridium strains
- Performed initial test cross with pLR418_Hinai Escherichia coli S17 and Clostridium
- Removed Clostridium origin of replication from pLR418
- Inserted mazF into pLR3.9
- Inserted evoglow\(^*\) into pLR3.9

**References**


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