

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.



Figure 1. Pictorial Representation of *Clostridium botulinum* with evoglow[®] replacing botulinum toxin

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 (BotNT) 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 BotNT in the past as a bioweapon; 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 [2]. 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 (FbFP) gene (EvoGlow-Clostridia, Evocat, 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.

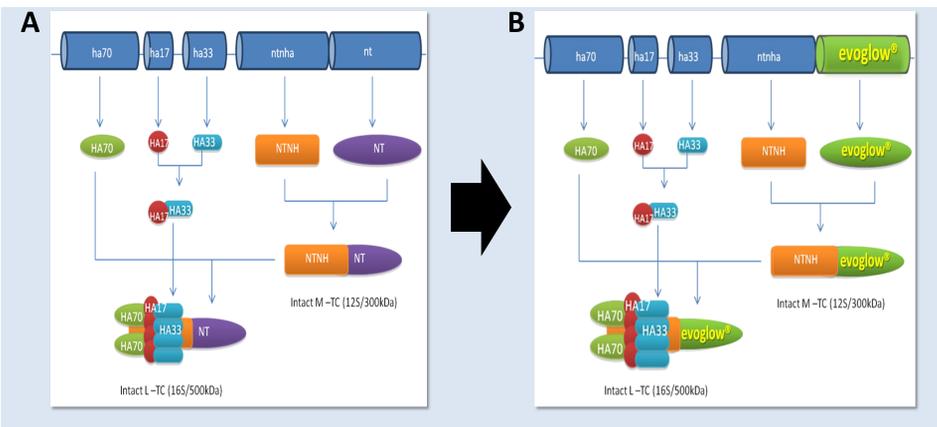


Figure 2. Model for botulinum toxin complex assembly pathway (figure adapted from Kouguchi[4]) For this project, the botulinum neurotoxin gene (nt) is replaced with the anaerobic functioning fluorescent protein evoglow[®] [Evocat, Dusseldorf, Germany].

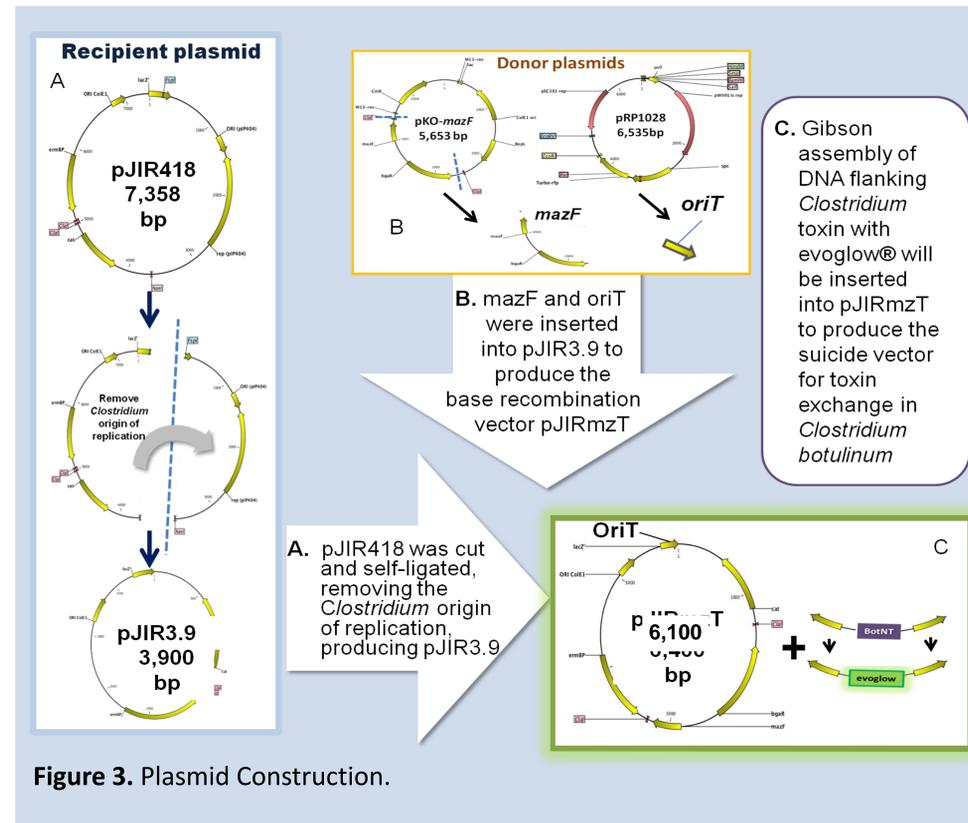


Figure 3. Plasmid Construction.

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 pJIRmzT, containing the genes for origin of plasmid transfer, origin of plasmid replication, as well as chloramphenicol resistance, the sequence specific mRNA endoribonuclease 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 induction of the *mazF* gene on the integrated plasmid whose expression is toxic to *Clostridia*.
- 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.

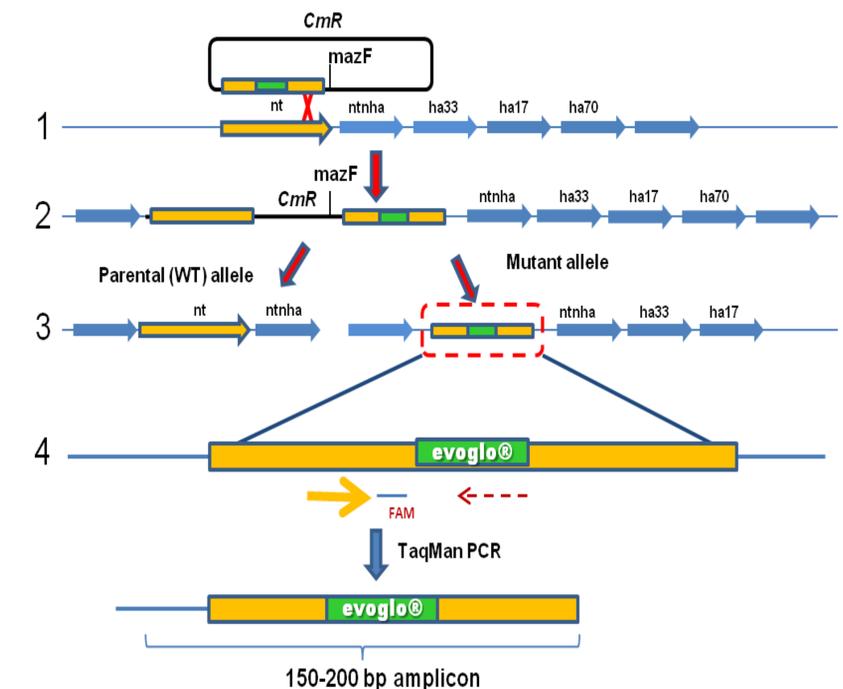


Figure 4: Engineering fluorescent protein evoglow[®] into *C. botulinum*:
 1. Homologous recombination of evoglow[®] into chromosome
 2. Select for antibiotic resistance
 3. Expression of *mazF* induces bacterial stasis
 4. Screen recombinants for desired strain using PCR to invariant region (dashed arrow)

Current Progress

- Anaerobic growth of *Clostridium* strains
- Performed initial test cross with pJIR418_oriT *Escherichia coli* S17 and *Clostridium*
- Removed *Clostridium* origin of replication from pJIR418
- Inserted oriT into pJIR3.9
- Inserted *mazF* into pJIR3.9

References

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