

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

Patricia E. Buckley, Ph.D.<sup>1</sup>, Sarah E. Katoski<sup>2</sup>, Henry S. Gibbons, Ph.D.<sup>1</sup>  
 (1) U.S. Army Edgewood Chemical Biological Center, (2) Excet, Inc.

## Abstract

Existing surrogates for spore-forming organisms are almost exclusively based on the *Bacillus* genus (e.g. *Bacillus atrophaeus*), which do not produce toxins in their sporulated state. For these organisms, the active product and the moiety of interest for detection and studies of dissemination is the spore itself. In contrast, toxin-producing clostridia (e.g. *Clostridium botulinum*, the causative agent of botulism) produce the potent botulinum neurotoxin (BoNT) during growth. For purposes of detection and decontamination, it is the toxin itself that is the active agent rather than the spores *per se*, making existing spore surrogates unable to adequately recapitulate the relevant properties of the active BoNT and its producing organism. Because of this, the development of surrogates for *C. botulinum* is highly desirable.

Because of its potency, *C. botulinum* strains and their associated toxins are regulated as CDC Tier 1 select agents and are highly restricted in their uses. Furthermore, the detection of the active species is non-trivial due to the proteinaceous nature of the toxin, which does not have an intrinsic and specific detection features such as fluorescence. For this reason, we propose 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. In some strains, a second fluorescent protein will be produced to mark the presence of spores.

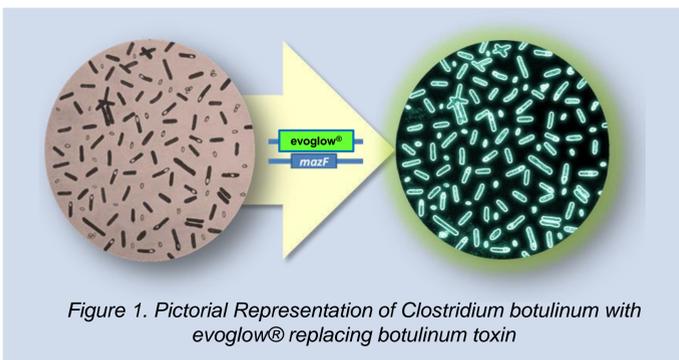


Figure 1. Pictorial Representation of *Clostridium botulinum* with evoglow® replacing botulinum toxin

## Background

There are many bacteria that are known to cause disease in humans, but there are few that produce as deadly an effect as *Clostridium botulinum*. The neurotoxins (BotNT) that are produced by *C. botulinum*, and some strains of *C. butyricum* and *C. baratii*, have been declared Category A agents on the scale for "Critical biological agent categories for public health preparedness" [1]. The category A agents on this list have the greatest potential for adverse public health impact, and have a moderate to high potential for large-scale dissemination, or the ability to invoke mass fear in the general population due to their heightened recognition to cause death [1]. Studies by the CDC have indicated that there is a relatively low incidence of naturally occurring outbreaks of botulism; therefore, a large outbreak could indicate 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.

## Background

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 newly developed genetic techniques for modifying *Clostridia*, we are replacing the protease domain of BoNT with a fluorescent protein reporter by homologous recombination using a new system developed by Al-Hinai *et al.* [2]. Because standard GFP-based reporter systems require oxygen for the formation of the fluorophore and thus will not work in obligate anaerobic systems, a flavin mononucleotide binding fluorescent protein (FbFP) gene (EvoGlow-Clostridia, Evocatal, GmbH; [3]) will be used in this effort. Understanding both the positive and negative attributes of botulinum toxin, as well as the formation of the toxin complex (Figure 2), may provide insight for developing medical countermeasures to protect the Warfighter and is necessary to identify targets for development of medical countermeasures, to predict exposure dose variations, and to inform battlefield risk assessment.

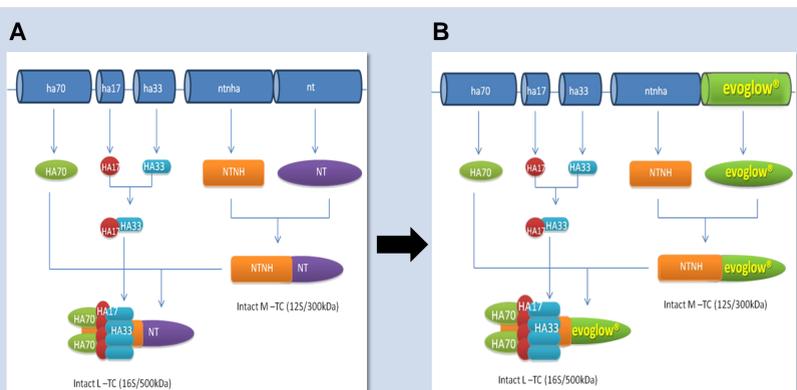


Figure 2. Model for botulinum toxin complex assembly pathway (figure adapted from Kouguchi[4])

**Toxin producing strain (Figure 2A).** The lower case letters indicate the gene in the chromosome, and the uppercase letters indicate the proteins produced. There are several genes located upstream of the toxin gene that form the hemagglutinin (ha) gene cluster. All toxin types associate with the non-toxic non-hemagglutinin protein (NTNH), and the toxin complexes are formed from the other proteins in the ha cluster. The labels in black indicate the toxins containing different proteins. The assembly pathway for each gene product and proteolytic pathway is indicated by solid arrows. There is no mutual conversion between the L and M toxin. *C. botulinum* Type A (used in this study) produces an LL toxin, which has different ratios of the proteins shown in this figure, as well as both the L and M toxins shown.

**Non-toxigenic reporter strain (Figure 2B).** The lower case letters indicate the gene in the chromosome, and the uppercase letters indicate the proteins produced. The labels in black indicate the toxins containing different proteins. The assembly pathway for each gene product and proteolytic pathway is indicated by solid arrows. For this project, the botulinum neurotoxin gene is replaced with the anaerobic functioning fluorescent protein evoglow® [Evocatal, Dusseldorf, Germany].

## 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.

## Methods Cont'd

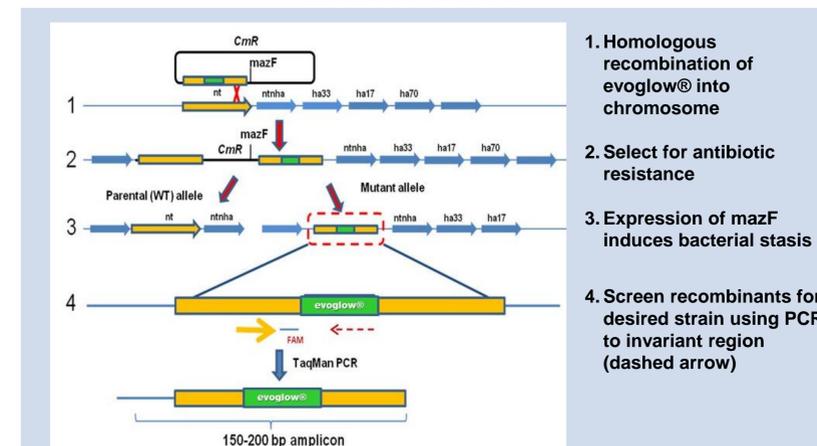


Figure 4. Engineering fluorescent protein evoglow® into *Clostridium botulinum*.

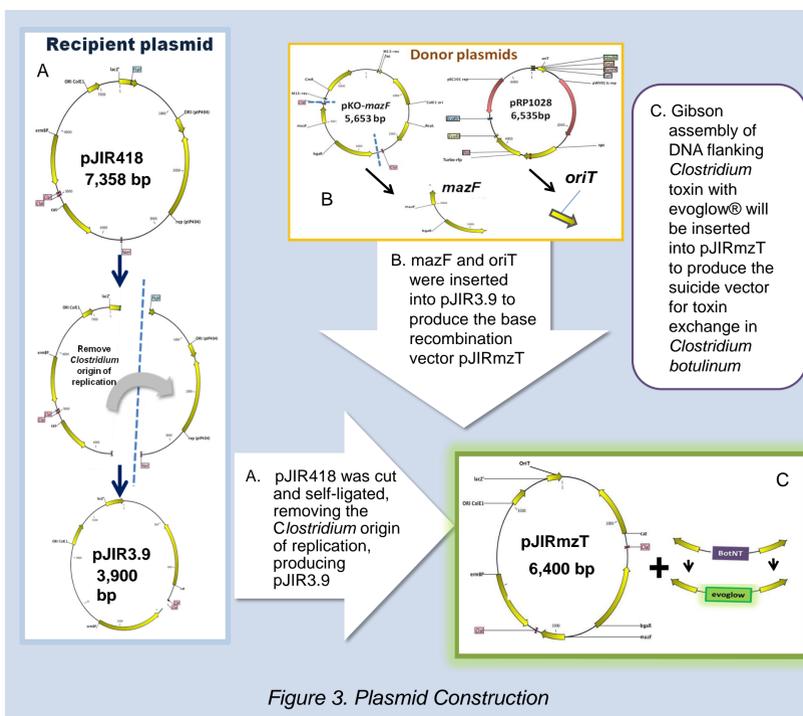


Figure 3. Plasmid Construction

## 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

- Rotz, L. D.; Khan, A. S.; Lillibridge, S. R.; Ostroff, S. M.; Hughes, J. M., Public health assessment of potential biological terrorism agents. *Emerging infectious diseases* **2002**, *8*, (2), 225-30.
- Al-Hinai, M.A., A.G. Fast, and E.T. Papoutsakis, *Novel System for Efficient Isolation of Clostridium Double-Crossover Allelic Exchange Mutants Enabling Markerless Chromosomal Gene Deletions and DNA Integration*. *Applied and Environmental Microbiology*, 2012. 78(22): p. 8112-8121.
- Drepper, T., et al., *Reporter proteins for in vivo fluorescence without oxygen*. *Nat Biotechnol*, 2007. 25(4): p. 443-5.
- Kouguchi, H., et al., *In vitro reconstitution of the Clostridium botulinum type D progenitor toxin*. *J Biol Chem*, 2002. 277(4): p. 2650-6.

Acknowledgements: The authors thank the Defense Threat Reduction Agency/Joint Science and Technology Office for their assistance and funding of this work. The views expressed in this presentation are those of the authors and do not necessarily reflect official policy or the position of the Department of Defense or the U.S. Government.



RDECOM

Approved for Public Release