Review of: "Construction and validation of safe Clostridium botulinum Group II surrogate strain producing inactive botulinum neurotoxin type E toxoid"

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Post-publication Review of 'Construction and validation of safe *Clostridium botulinum* Group II surrogate strain producing inactive botulinum neurotoxin type E toxoid'

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The paper 'Construction and validation of safe *Clostridium botulinum* Group II surrogate strain producing inactive botulinum neurotoxin type E toxoid' summarizes a collaborative study led by the group of Miia Lindström and five additional PIs (Birgitte Dorner, Martin Dorner, Rongshen Jin, Nigel Minton, and Andreas Rummel) who are established in the *Clostridium botulinum* (*C. botulinum*) field. The paper is well-written and describes the use of CRISPR-Cas technology to create a mutated *C. botulinum* strain. The CRISPR-Cas technology has only recently been adapted to this organisms, in large part thanks to the efforts of Nigel Minton, who developed the CRISPR-Cas plasmids specifically designed for *Clostridia*. Few available gene editing methods are available for *C. botulinum*, which is a Tier 1 Select Agent in the US and thus highly regulated, resulting in only few labs working with this organism. In addition, the low GC content, the gram positive cell wall, and the polyphyletic nature of this 'species' complicates genetic manipulations. Utlization of CRISPR-Cas technology to a *C. botulinum* strain for site directed mutagenesis in specific genes is thus highly significant and novel.

The gene that was targeted for mutation in this study was the botulinum neurotoxin E (*bont/E*) gene, introducing three single amino acid mutations that have previously been shown to disrupt catalytic activity by preventing zinc binding and transition state stabilization of the toxin's light chain. This resulted in an attenuated *C. botulinum* strain that has many potential utilities. Additionally, three silent mutations were included in the same gene near the mutation site to add three unique restriction enzyme recognition sites for easy identification of mutants. This is an elegant approach, provided codon usage of the organism is considered when designing the silent mutations, such that translation efficiency is not altered.

Describing the strain as safe and the toxin as inactive is a slight overstatement, as the safety of the strain still remains to be determined in future studies and is dependent on its use, and based on the literature this toxin should be described as attenuated not inactive as it has not been isolated and tested at higher concentrations.

The authors clearly describe the significance of the created attenuated *C. botulinum* Ei strain in the introduction and

discussion, and appropriately discuss the advantages this strain poses compared to non-toxic *C. botulinum* species for food safety studies. However, to completely understand the value of this strain in food challenge studies, a discussion of the mouse bioassay and other currently used assays and regulatory requirements is needed. Currently, in the USA an ELISA can be used for food challenge studies but results have to be confirmed with the mouse bioassay. Additionally, a BoNT ELISA needs to be validated for the food matrices it is used with. Thus an attenuated strain expressing a catalytically inactive toxin could be used to directly evaluate toxin production in specific food matrices, provided the mutated toxin has equal stability in various matrices compared to wild type toxin. Finally, additional growth, spore survival, and germination studies must be conducted under various conditions, to ensure this strain behaves similar to the wt strain under a variety of growth conditions, as would be encountered in food challenge studies. In spite of these restrictions on immediate utility of this strain, the authors emphasize that this method provides a blueprint for future production of additional attenuated *C. botulinum* strains, which is a critically important point to consider for construction of a food challenge strain cocktail, as is commonly used for *C. botulinum* food challenge studies. As *C. botulinum* is a polyphyletic species with significant differences in specific strain characteristics, strain selection for this project will be of paramount importance.

Other important aspects of this study include the utility of this approach to create additional mutated strains to enable studies of involvement of specific genes in key metabolic processes in this organism including sporulation, germination, nutrient requirements, and regulation of toxin production. Such studies have so far been very challenging with this organism due to the limited availability of genetic tools. Specific mutations of the toxin gene, such as removing catalytic activity as in this study, will also enable future studies on the significance of the toxin gene (function) for the organism, an as yet un-answered central question in understanding this virulence factor. The *C. botulinum* Ei strain constructed here also has great utility for structural studies, as the authors point out.

One short-coming of the presented study is the lack of discussion of genome sequencing of the created mutated strain. It is apparent from the methods and supplementary data that the authors conducted whole genome sequencing, and that a relatively large number of mutations were detected between the wild type and the mutated strain. It is unclear from the study how many passages were required to remove the CRISPR-Cas9 vector in the mutant strain, and only the mutant strain, not the wt strain, was assessed at G25. As a result, it is undetermined whether these additional mutations at G25 occurred before or after the removal of the CRISPR-Cas9 vector and whether they occurred due to the direct activity of the CRISPR-Cas9 vector while present within the Beluga Ei strain, or a lasting hyper-mutator phenotype triggered by the CRISPR-Cas9 vector prior to it being cured. Knowing whether the additional mutations observed in the Beluga Ei strain arose prior to or following curing of the CRISPR-Cas9 vector is important in regards to the utility and safety of the attenuated strain as a surrogate in food studies. Provided the stated purpose of the strain for use in food studies, assessment of the additional mutations in the Beluga Ei strain and their possible effects on physiology seems quite important. Further, inclusion of a discussion of additional and alternative approaches for strain construction for food challenge studies would be much appreciated, including production of strains with tagged (catalytically inactive) toxin for easier detection. A further consideration is whether the number of mutations between G1 and the G25 indicate genetic instability of the strain, which in turn would raise questions regarding the safety of the strain. The authors suggest that the strain is only used in ‘contained’ research facilities, yet it has received exemption from the US federal select agent

program as an attenuated *C. botulinum* strain, and thus is not regulated. So it is unclear how this can be controlled.

It is very interesting to note that the US Federal Select Agent program has exempted this strain from the Select Agent list as an attenuated strain, based on the evidence that the BoNT/Ei toxiciuty by mouse bioassay is at least 23,000-fold reduced compared to wild type BoNT/E. Based on the literature, even recombinant BoNTs for which *in vitro* assays did not detect residual catalytic activity, retain some *in vivo* toxicity [1]. Such residual toxicity would not have been detected in the current study, as mice were injected with only about 1-2 µg of BoNT/Ei (trypsinized), and with other attenuated BoNTs *in vivo* toxicity is observed with 10-100-fold greater amounts. In addition, the ELISA method of toxin quantification may not be accurate, especially considering the observed BoNT/E mouse LD50 dose was 2-3 times lower than is usually observed for purified BoNT/E [2]. Thus, while the relative estimate of at least 23,000-fold reduced toxicity remains the same, the absolute amount injected into mice may actually have been below 1 µg/mouse. In order to determine remaining toxicity of BoNT/Ei, the toxin will need to be isolated and tested in mice, and ideally also in neuronal cells to confirm absence of all catalytic activity within the neuronal cell cytosol. However, even if some toxicity remains, the great attenuation in toxicity of BoNT/Ei compared to BoNT/E wt significantly reduces risks associated with this strain and toxin. There currently is no definition on potency required for a BoNT or BoNT-like homolog or a strain expressing them to be considered Select Agent, and the exemption of this strain as an attenuated strain is novel. Interestingly, BoNT/A4, which is 1,000-fold less potent than BoNT/A1 (just 2.3-fold less difference than shown in this paper for BoNT/Ei versus BoNT/E), is still considered a Select Agent. The relatively recently identified BoNT/X, for which it has been demonstrated that culture supernatants are not toxic to mice similar to the strain constructed here, was deemed of sufficient risk to be regulated as Select Agent toxin.

Finally, the current study appears to use a truncated Cas9, a nickase, for the generation of this mutant strain. Nickases are known to result in increased off-target effects [3]. Since the first construction of CRISPR-Cas9 systems for *C. botulinum* by Nigel Minton, several improvements have been made. A discussion on the challenges of CRISPR-Cas systems in *C. botulinum*, reasons for choosing a nickase here, recent improvements in general and particular for *C. botulinum*, and ability to transfer the technology to other strains of *C. botulinum*, would have been much appreciated as part of this article. This technology will no doubt transform research in the *C. botulinum* field, as exemplified by this highly significant paper.

References:

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