Mechanisms underlying the transfer of plasmid-borne BoNTs in Group I *C. botulinum*

By

Erin M. Nawrocki

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The dissertation is approved by the following members of the Final Oral Committee:

Eric A. Johnson, Professor, Bacteriology
Marcin Filutowicz, Professor, Bacteriology
Jue Wang, Professor, Bacteriology
John-Demian Sauer, Assistant Professor, Medical Microbiology & Immunology
Jan Peter van Pijkeren, Assistant Professor, Food Science

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Abstract

Botulinum neurotoxins (BoNTs), once thought to be exclusive to the species *Clostridium botulinum*, are among the world's most potent toxins. The discovery of novel BoNTs, the presence of botulinum-like toxins outside *C. botulinum*, and the genomic diversity of the species itself all suggest that the *bont* gene may be subject to horizontal gene transfer. While most Group I *Clostridium botulinum* strains harbor *bont* genes on their chromosome, some carry these genes on large plasmids. Prior work in our laboratory indicated that Group I BoNT plasmids could be mobilized to *C. botulinum* recipient strains that contain the Tn*916* transposon. Here, we show that Tn*916* is nonessential for plasmid transfer. Relying on an auxotrophic donor strain and a plasmid-borne selectable marker, we demonstrate the transfer of pCLJ, a 270 kb plasmid harboring two *bont* genes, from its native Group I *C. botulinum* strain to various clostridia. pCLJ transfers with the highest frequency to other Group I *C. botulinum* recipients. It can also be transferred into traditionally nontoxigenic species such as *C. sporogenes*, where the minor plasmid-borne toxin BoNT/A4 is expressed and active.

In addition, we identify a gene on pCLJ that is necessary for the plasmid's transfer. Using the ClosTron method of insertional inactivation to mutagenize plasmid genes and a derivative of the ClosTron to tag a representative recipient strain with a second selectable marker, we show that the inactivation of CLJ_0213 is not permissive to conjugation. The conjugation defect can be partially rescued by complementation with a copy of CLJ_0213 in *trans*. CLJ_0213, a putative ATPase, is the first gene outside the toxin complex on pCLJ to be functionally characterized. The identification of a gene essential for pCLJ conjugation aids in defining the molecular mechanism of plasmid transfer in *C. botulinum*. Taken together, these data indicate that conjugation within the genus *Clostridium* can occur across physiological Groups of *C. botulinum* and that CLJ_0213 plays an essential role in the conjugative process.

Background and Significance

Clostridium botulinum *and its neurotoxins*

The genus *Clostridium* is composed of gram-positive, spore-forming, anaerobic bacteria. Several members are pathogens, causing diseases such as tetanus (*C. tetani*), food poisoning and gas gangrene (*C. perfringens*), diarrhea (*C. difficile*), and botulism (*C. botulinum*). *C. botulinum* is notable for its production of botulinum neurotoxin (BoNT). Besides being a deadly toxin, BoNT is also a common pharmaceutical agent with many therapeutic and cosmetic applications. BoNTs are zinc-dependent proteases that specifically cleave SNARE proteins, and in doing so prevent the release of neurotransmitters from synaptic vesicles (1). Intoxication with BoNT results in flaccid paralysis; if untreated, death by respiratory failure ensues (2).

Clinical botulism cases are categorized into six forms, depending on their route of acquisition: foodborne, wound, infant, intestinal, inhalational, and iatrogenic (2). Foodborne and iatrogenic botulism are caused by the ingestion and injection, respectively, of pre-formed BoNT complexes. Inhalational botulism is caused by BoNT intoxication through the respiratory route but does not occur naturally. Wound, infant, and intestinal botulism are all caused by the introduction of *C. botulinum* spores to a suitable environment in humans and animals. The spores may germinate in the anaerobic conditions of a wound or the gastrointestinal tract, reproduce, and release toxin into the bloodstream. This route of infection typically requires a particular susceptibility of the host, such as an altered gut microbiome, intense antibiotic treatment, or immunosuppression. Infants up to a year of age are predisposed because they lack a community of competitive intestinal bacteria, which in the normal adult microbiome are inhospitable to the proliferation of *C. botulinum* (3, 4).

Botulism is characterized by flaccid paralysis that lasts from days to months, or even up to a year in very severe cases. Death appears to be a result of paralysis of the respiratory musculature. No cure exists, but early on after intoxication and before the toxin has entered the nervous system, antitoxin (polyclonal equine antisera) can be used to neutralize the toxin and reduce the severity and duration of botulism. Beyond this pharmacologic intervention, intensive therapy including respiratory support is the only medical intervention available. Because of these factors and the ultimate potency of BoNTs, *C. botulinum* and its neurotoxins are considered potential weapons of bioterrorism, and consequently, toxigenic *C. botulinum* strains are classified as Tier 1 Select Agents by the Department of Health and Human Services and the Centers for Disease Control and Prevention (5, 6). This classification has restricted much research on this organism and toxin, and only a few labs in the United States have the regulatory approvals and expertise to conduct studies with these agents.

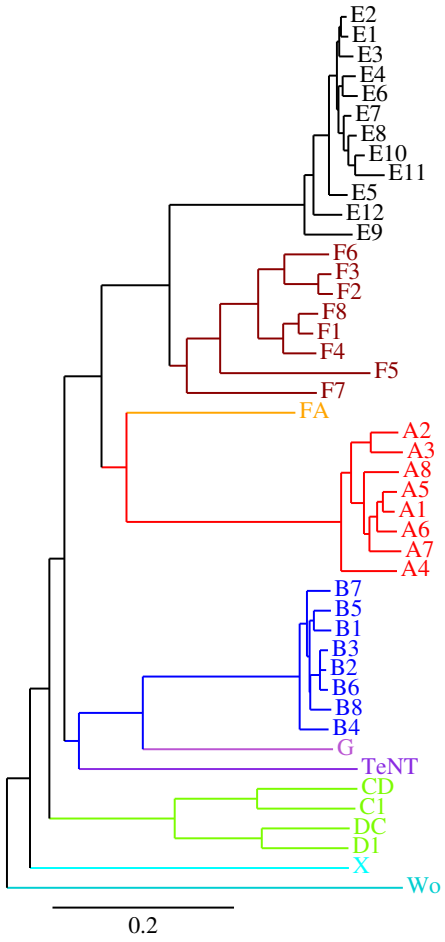

Figure 1. The current BoNT serotypes form discrete clades. The protein sequences of representative BoNT subtypes from each serotype were aligned to each other using the default parameters of Clustal Omega. Included are sequences of tetanus neurotoxin (TeNT), the hybrid BoNT/FA, and the putative novel toxins BoNT/X and BoNT/Wo. Figure kindly provided by Sabine Pellett.

Clostridial taxonomy and phylogeny

BoNTs exist in seven serotypes, designated A through G on the basis of their neutralization with homologous antisera. Within the serotypes, neurotoxins are further classified into subtypes based on amino acid similarity, with genes attaining new subtype status if they

differ from published *bont* sequences by 2.6% or more (7; Figure 1). Even considering immunological distinctions, there can be a vast difference in the activity of neutralizing antibodies against the subtypes within a serotype (8). BoNTs are synthesized as single-chain 150 kDa polypeptides that are then nicked into a more toxic dichain form (9). Most BoNT-producing strains encode a single neurotoxin, but some have a second "silent" BoNT gene that does not produce active toxin due to a premature stop codon. Still others encode and produce two or even three active neurotoxins in varying quantities (10).

The diverse *Clostridium* strains expressing the various BoNT serotypes are classified into four physiological Groups, designated with numerals I-IV. Group I *C. botulinum* strains are termed proteolytic, as they encode extracellular proteases for acquiring amino acid nutrients and for nicking the single chain BoNT molecule; these strains have an optimum growth temperature of 37°C and can produce BoNTs of serotypes A, B, and F (10). Group II strains are termed nonproteolytic: they do not encode their own nicking protease, but rather rely on proteolytic enzymes in the environment to convert their neurotoxins to the active dichain form. They prefer lower temperatures (30°C) and higher water activity for growth and toxin formation, and they may produce BoNTs of serotypes B, E, and F (10). Groups I and II are most commonly responsible for human botulism cases, and therefore contain the most extensively researched strains, including those studied in this project.

Group III and Group IV strains are also of interest with regards to horizontal gene transfer, as their toxins are found in association with bacteriophages and plasmids, respectively. Group III *C. botulinum* strains produce BoNT/C, BoNT/D, or chimeric toxins (BoNT/CD or BoNT/DC) that appear to be hybrids of the two serotypes. These serotypes are encoded on large bacteriophages that propagate extrachromosomally; they cause avian and nonhuman mammalian

botulism (11). Finally, Group IV strains produce BoNT/G, which is typically plasmid-encoded and has been implicated in rare human intoxications (12).

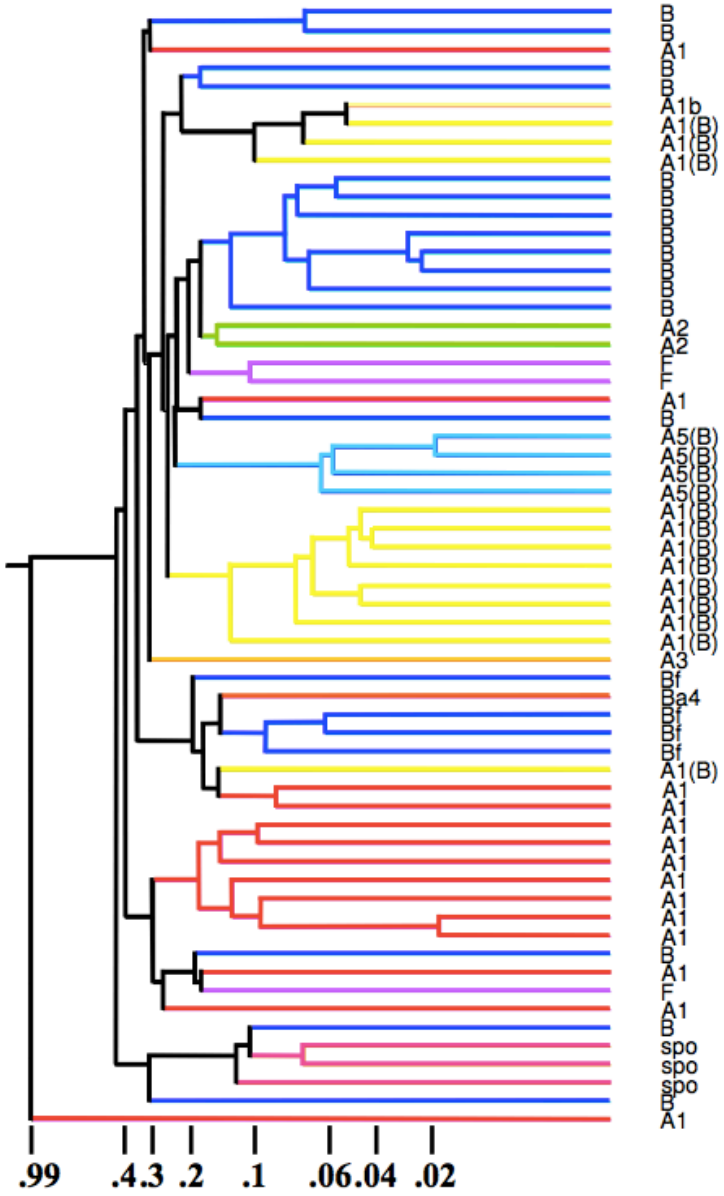

Figure 2. BoNT serotype is not correlated with strain phylogeny. Toxin types are not organized phylogenetically among Group I *C. botulinum* strains but rather are distributed among various genetic backgrounds. Adapted from Carter et al. 2009.

Historically, BoNT production was the sole criterion required for designation of the species *C. botulinum*, a framework that has resulted in the complicated phylogeny of the species today. The availability of sequence information has not only revealed the great genetic distance between the four Groups, but also has enabled the characterization of BoNT-producing strains of other clostridial species, namely *C. butyricum* and *C. baratii*. Intriguingly, a single *C. botulinum* Group can produce multiple toxin serotypes, and likewise, a single serotype can be produced by strains in multiple Groups. Several strains of *C. botulinum* produce more than one serotype of BoNT in culture. Studies have shown that *C. botulinum* strains and BoNTs have

evolved independently of one another, suggesting that horizontal gene transfer may be responsible for toxin acquisition (13-15). The discrepancy between strains and toxin serotypes is partly illustrated in Figure 2; genomes have been aligned by microarray (not shown) and are color-coded by the serotype of toxin they produce (13).

Neurotoxin gene clusters

All *bont* genes are encoded as part of a conserved multigene cluster, where they are found alongside genes encoding the so-called accessory proteins. Two such clusters exist: *ha* (hemagglutinin) and *orfX*, each of which is associated with multiple toxin subtypes. An example of each is shown in Figure 3. The *ha* cluster is better studied, as its accessory proteins have been crystallized with BoNT/A (16). HA70, HA17 and HA34, co-transcribed and named on the basis of their molecular weight, are thought to help stabilize the neurotoxin in the gastrointestinal tract and upon crossing the intestinal epithelium (17). In strains with the *orfX* cluster, the OrfX proteins (OrfX3, OrfX2, and OrfX1) replace the HAs, but little is known about their function (14). In either case, the cluster has a second transcriptional unit comprising the *bont* gene and *ntnh*, or non-toxic non-hemagglutinin. BoNT and NTNH share similar domain architecture and are presumed to have arisen from a gene duplication; NTNH forms a tight, interlocking complex with BoNT, protecting it from degradation in the intestines (18, 19; Figure 4).

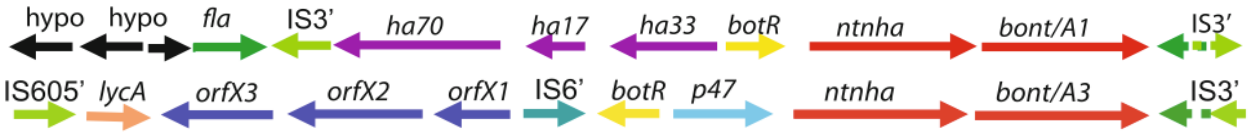

Figure 3. Two types of toxin gene clusters are present in *C. botulinum*. Hemagglutinin (HA) or OrfX proteins and nontoxic non-hemagglutinin (NTNHA) accessory proteins are transcribed from genes in this cluster and form a complex that stabilizes the BoNT. The cluster is flanked by partial IS elements and other associated genes. BotR is a transcriptional regulator, not discussed in this work. Adapted from Hill and Smith 2013.

In most sequenced strains, *bont* gene clusters are not randomly dispersed throughout the genome, but instead are located in particular positions. For example, among some Group I *C. botulinum* strains, *bont/a1*, *bont/a2*, and *bont/f1* are located in an *orfX* cluster that is inserted in the *arsC* operon, adjacent to arsenate reductase genes (20). Other Group I strains contain *bont/a1*, *bont/(b)*, or *bont/a5* in a *ha* cluster within the *oppA/brnQ* operon (20). Strains with plasmid-encoded BoNTs contain toxin clusters at conserved loci on the plasmid (20). These are not the only possible loci for BoNT genes, however. Intriguingly, Group II strains contain *bont/e* in an *orfX* cluster that has inserted itself into a *rarA* gene, coding for a transposon-associated resolvase. The locus consists of a *rarA* homologue split in two by the toxin cluster and other associated DNA, including an intact *rarA* gene and IS elements. The mechanism of this insertion is not known, but it is possible that the functional copy of *rarA* located the homologue, split it, and inserted itself along with the toxin cluster (20, 21). All known Group II BoNT/E and /F strains follow a similar scheme, in which the toxin cluster is inserted into a gene associated with DNA recombination (14).

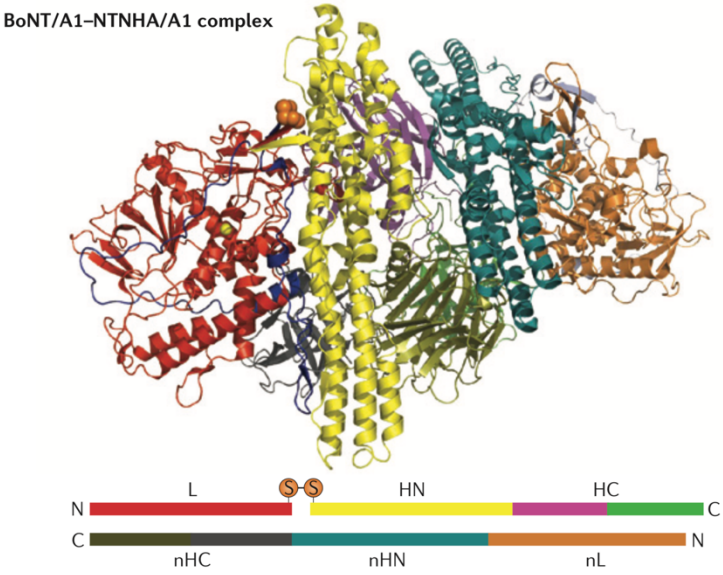

Figure 4. Botulinum neurotoxin and its nontoxic non-hemagglutinin protein form a tight complex. The two proteins have the same domain organization and were cocrystallized, PDB accession number 3V0B. Adapted from Rossetto et al. 2014.

The mobility of BoNT genes is not a novel concept. Early sequence analyses of the toxin clusters and their flanking regions noted the presence of elements involved in recombination, namely flagellin genes and IS elements (22). Intact or partial flagellin genes are present

upstream of the toxin gene cluster in some strains and are frequently hypervariable sites of recombination in other species (23-26). IS elements of various families can be found on both sides of the toxin cluster in *C. botulinum*, both plasmid-borne and chromosomally. When intact, IS elements encode transposase enzymes that promote their own translocation; those near the toxin genes appear to be impaired, sharing up to 83% homology with the full-length elements (27). Finally, convincing evidence of mobility is seen within the toxin cluster itself: the *ntnh* gene appears to be a hot spot for recombination, as seen in strains with *ntnh* variants that are hybrids between those of serotypes B and A (21). Recombination at this site led to the presence of a *bont*/*a1* gene in a toxin cluster usually associated with *bont*/*b* and could promote diversity in other *C. botulinum* strains by driving gene exchange between serotypes (21). In one striking example of recombination involving the *bont* gene cluster, a Group II *C. botulinum* strain with an ancestral *bont*/*b* gene had its toxin cluster disrupted first by a *bont/e* cluster before acquiring the *bont/f* cluster it retains today (28). This unusual strain possesses the remnants of IS elements, site-specific recombinases, and bacteriophage integrases flanking its toxin gene, suggesting a plethora of possible mechanisms by which BoNTs may be incorporated into the genome.

Plasmids in C. botulinum

Even as evidence mounts for the mobility of toxin gene clusters, the question as to their dissemination remains. In other words, how might a strain of *Clostridium* acquire a new toxin gene cluster and the ability to produce active BoNT complexes? Plasmids are a likely suspect. The observation that some Group I strains encoded their toxins on plasmids came as somewhat of a surprise (29). Prior to this, it was thought that *bont* loci were characteristic of their serotype (i.e. A, B, E, and F on the chromosome; C and D on bacteriophages; G on a plasmid). In the years since, as BoNT-encoding plasmids have been sequenced, the *bont* genes have been found

in new genomic sites, but the same genes and elements described above often flank them. Thus, it seems likely that toxin clusters may be able to move between chromosomes and plasmids in *C. botulinum*.

Unfortunately, the BoNT-encoding plasmids are poorly understood. In Group I strains, they range from approximately 140-270 kb in size and encode toxins of serotypes A, B, F, or multiple toxins (27, 30, 31). The plasmids within proteolytic strains of *C. botulinum* share homology with each other, but they are not similar to any other known clostridial toxin-encoding plasmids, including those of *C. tetani* and *C. perfringens* (27). Peculiarly, these BoNT-encoding plasmids contain neither the prototypical *E. coli*-type rolling-circle replication genes nor the *B. anthracis*-type theta replication genes, and so their mechanism of replication is unknown (27). They do seem to replicate independently, however, as they encode intact DNA polymerase III and DNA helicase II enzymes, and these are highly conserved among the sequenced BoNT-encoding plasmids of Group I strains (31). Group I toxin plasmids have about 25% GC content, which is just slightly lower than the median GC content (28%) of *C. botulinum* as a whole (32). Note that although relatively few completed sequences are available, the presence of BoNT genes on plasmids could be widespread—one survey found *bont*/*b* on a plasmid in 32 of 60 strains examined, including 21 of 22 *bont*/*b1* strains (30).

The current significance of plasmid-borne toxin genes, of course, depends on their ability to be mobilized. Movement of BoNT-encoding plasmids into nontoxigenic clostridial strains, particularly species with more resistant phenotypes, would pose real threats to health and safety, especially given the wide prevalence of clostridia in the environment and in the mammalian gastrointestinal tract. A recent study confirms this possibility, showing similar plasmids harboring *bont*/*b6* present in strains of both the *C. botulinum* and *C. sporogenes* chromosomal lineages (15). Multi-domain homologues of BoNTs were also newly identified in several non-

clostridial genomes, including strains of *Weissella oryzae*, *Chryseobacterium piperi*, and *Enterococcus faecium*, raising questions about the origin and distribution of the neurotoxin (33-36). While plasmid transfer is the focus of this work, it is not the only mechanism of horizontal gene transfer. There are numerous examples of mobile genetic elements including bacteriophages, integrons, conjugative transposons, and plasmids (37). *C. botulinum* and its toxin gene clusters were likely shaped by many of these elements, which would be frequently encountered in the soil and intestinal microbiota.

As with the mechanism of replication, the mechanism(s) of BoNT-encoding plasmid mobilization is yet to be determined. About 8 years ago in our laboratory, experiments by Marshall et al. suggested that certain BoNT-encoding plasmids (ranging from ~50 kb to ~270 kb) are conjugative, as evidenced by the necessity of cell-to-cell contact for their transfer (38). However, the genes required for transfer were not identified. Moreover, the recipient strain used in the studies contained a Tn*916* transposon, which may have affected plasmid transfer. The Group I *C. botulinum* plasmids do not contain a functional *tcp* (transfer clostridial plasmids) locus, which is conserved in all conjugative *C. perfringens* plasmids (38, 39; Figure 5). There are, however, several interesting candidate ORFs on the BoNT-encoding plasmids whose products are annotated as pilus assembly proteins and ATPases involved in coupling or substrate translocation that may permit conjugation by a novel mechanism (31, 38, 40; Figure 6).

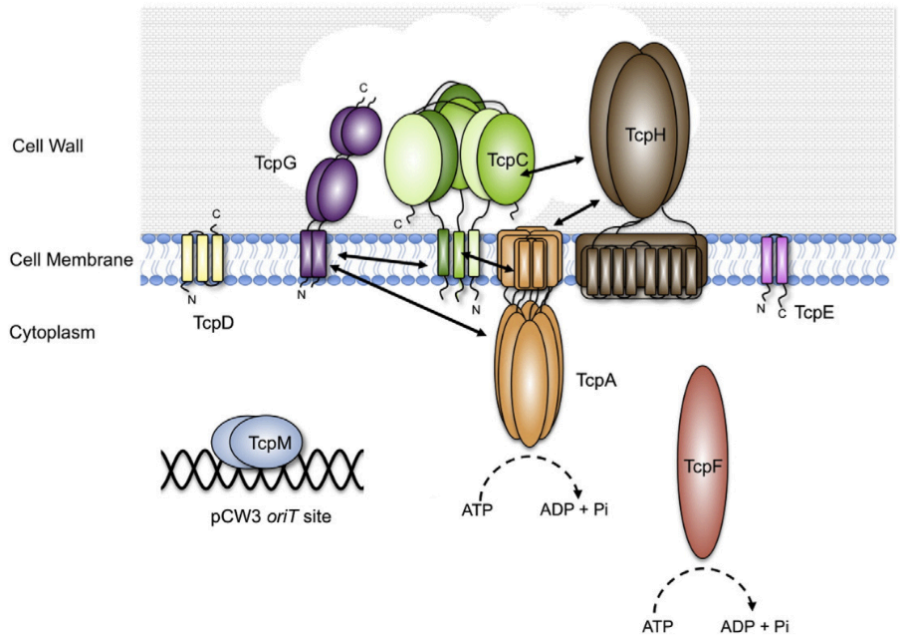

Figure 5. The *C. perfringens tcp* locus is a model of clostridial conjugation. *tcp* spans 11 genes on the prototypical tetracycline resistance plasmid pCW3. Proteins required for conjugative transfer of the plasmid and their subcellular localizations are shown here. Protein-protein interactions are indicated with solid black arrows. Reproduced from Wisniewski et al. 2017.

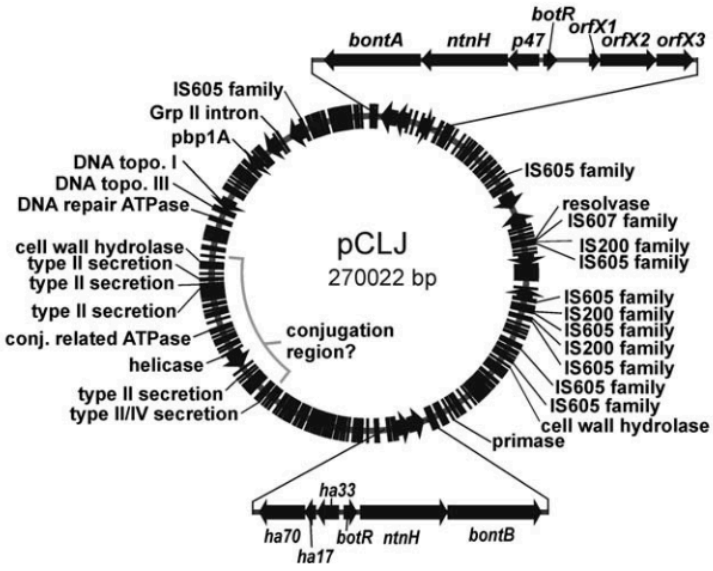

Figure 6. pCLJ encodes two BoNTs and a "conjugation region." Toxin gene clusters are noted at top and bottom. The conjugation region includes a relevant ATPase and various secretion system components. Adapted from Adams et al. 2014.

Molecular genetics of C. botulinum

At present, our ability to achieve genetic manipulations in *C. botulinum* is limited. Most mutants in the contemporary literature are those with genes inactivated by insertion of the ClosTron, which was introduced for use in *Clostridium* in 2007 (41). This system involves a group II intron that is guided to a specified target sequence by retrohoming, whereby it integrates into the chromosome (42). Upon this integration, the retrotransposition-activated marker (RAM) becomes active and confers antibiotic resistance on the cell (41). To assure that the intron is stably integrated, one must confirm the loss of the plasmid vector, which encodes the protein responsible for intron splicing (43). This

technology has its drawbacks—it requires a suitable site for insertion, restricts the antibiotic resistance markers to only those genes that the RAM can accommodate, and is unable to completely remove target genes.

Alternative strategies have been used successfully in other clostridia. One group coupled the ClosTron method to a site-specific recombinase, allowing for the removal and recycling of the *ermB* antibiotic resistance marker and the subsequent creation of a double mutant *C. acetobutylicum* strain (44). Allelic exchange systems using MazF and PyrE as markers have been employed in *C. acetobutylicum* and *C. difficile*, respectively (45, 46). *C. acetobutylicum* has also been modified by single-stranded DNA recombineering, namely by inducing a recombinase protein from *C. perfringens* and electroporating the cell with an oligonucleotide to introduce a mutation conferring erythromycin resistance (47).

If these mutagenesis strategies are functional but inefficient, they may be combined with more stringent selection by the CRISPR-Cas9 system. Several reports from other bacteria show that recombineering efficiency approaches 100% when CRISPR-Cas9 is used to select against unedited cells; these studies also show that oligonucleotides may be used to introduce chromosomal deletions of up to 1 kb, while dsDNA PCR products can be used to extend that range to approximately 19 kb (48, 49). To bypass this dual-method implementation in *C. beijerinckii*, one group chose to place a DNA editing template on the same vector as the CRISPR-Cas9 system. The plasmid-encoded template integrated into the clostridial genome by a double-crossover event, and in doing so caused a deletion of 262 bp (50). This all-in-one approach was also applied to *C. cellulolyticum*, where templates encoding both insertions and deletions were successfully incorporated via a plasmid encoding a Cas9 nickase (51). In each of these cases, the desired mutation produced a selectable phenotype, such as distinct colony morphology or resistance to a particular chemical. Mutants were therefore obtained by a double

selection for cells that had obtained the plasmid (conferring antibiotic resistance) and integrated the editing template (conferring the phenotype). Despite its potential limitations, this strategy is an attractive one, as it allows the editing template to be retained for as long as the plasmid replicates (51).

Many techniques, including the ClosTron, that were pioneered in other clostridial species were later extended to *C. botulinum*. Given the amenability of *C. acetobutylicum*, *C. difficile*, *C. beijerinckii*, and *C. cellulolyticum* to manipulation with CRISPR-Cas9 editing and other tools, *C. botulinum* may soon lend itself to efficient genetic manipulation as well.

Works Cited

1. Rossetto O, Megighian A, Scorzeto M, Montecucco C. Botulinum neurotoxins. Toxicon. 2013;67:31-6. Epub 2013/02/26. doi: 10.1016/j.toxicon.2013.01.017. PubMed PMID: 23435262.
2. Johnson EA, Montecucco C. Botulism. Handbook of clinical neurology. Vol. 91 ed2008. p. 333-68.
3. Arnon SS, Damus K, Chin J. Infant botulism: epidemiology and relation to sudden infant death syndrome. Epidemiol Rev. 1981;3:45-66. Epub 1981/01/01. PubMed PMID: 7030764.
4. Koepke R, Sobel J, Arnon SS. Global occurrence of infant botulism, 1976-2006. Pediatrics. 2008;122(1):e73-82. Epub 2008/07/04. doi: 10.1542/peds.2007-1827. PubMed PMID: 18595978.
5. Federal Select Agent Program. Select Agents and Toxins List. 2014.
6. Arnon SS, Schechter R, Inglesby TV, Henderson DA, Bartlett JG, Ascher MS, Eitzen E, Fine AD, Hauer J, Layton M, Lillibridge S, Osterholm MT, O'Toole T, Parker G, Perl TM, Russell PK, Swerdlow DL, Tonat K, Working Group on Civilian B. Botulinum toxin as a biological weapon: medical and public health management. JAMA. 2001;285(8):1059-70. Epub 2001/03/07. PubMed PMID: 11209178.
7. Peck MW, Smith TJ, Anniballi F, Austin JW, Bano L, Bradshaw M, Cuervo P, Cheng LW, Derman Y, Dorner BG, Fisher A, Hill KK, Kalb SR, Korkeala H, Lindstrom M, Lista F, Luquez C, Mazuet C, Pirazzini M, Popoff MR, Rossetto O, Rummel A, Sesardic D, Singh BR, Stringer SC. Historical Perspectives and Guidelines for Botulinum Neurotoxin Subtype Nomenclature. Toxins. 2017;9(1). Epub 2017/01/21. doi: 10.3390/toxins9010038. PubMed PMID: 28106761; PMCID: PMC5308270.
8. Montecucco C, Rasotto MB. On botulinum neurotoxin variability. MBio. 2015;6(1). Epub 2015/01/08. doi: 10.1128/mBio.02131-14. PubMed PMID: 25564463; PMCID: PMC4313909.
9. DasGupta BR. Botulinum neurotoxins: perspective on their existence and as polyproteins harboring viral proteases. J Gen Appl Microbiol. 2006;52(1):1-8. Epub 2006/04/07. PubMed PMID: 16598153.
10. Peck MW, Stringer SC, Carter AT. *Clostridium botulinum* in the post-genomic era. Food Microbiol. 2011;28(2):183-91. Epub 2011/02/15. doi: 10.1016/j.fm.2010.03.005. PubMed PMID: 21315972.
11. Skarin H, Hafstrom T, Westerberg J, Segerman B. *Clostridium botulinum* group III: a group with dual identity shaped by plasmids, phages and mobile elements. BMC Genomics. 2011;12:185. Epub 2011/04/14. doi: 10.1186/1471-2164-12-185. PubMed PMID: 21486474; PMCID: PMC3098183.
12. Zhou Y, Sugiyama H, Nakano H, Johnson EA. The genes for the *Clostridium botulinum* type G toxin complex are on a plasmid. Infect Immun. 1995;63(5):2087-91. Epub 1995/05/01. PubMed PMID: 7729925; PMCID: PMC173270.
13. Carter AT, Paul CJ, Mason DR, Twine SM, Alston MJ, Logan SM, Austin JW, Peck MW. Independent evolution of neurotoxin and flagellar genetic loci in proteolytic *Clostridium botulinum*. BMC Genomics. 2009;10:115. Epub 2009/03/21. doi: 10.1186/1471-2164-10-115. PubMed PMID: 19298644; PMCID: PMC2674064.
14. Carter AT, Peck MW. Genomes, neurotoxins and biology of *Clostridium botulinum* Group I and Group II. Res Microbiol. 2015;166(4):303-17. Epub 2014/12/03. doi: 10.1016/j.resmic.2014.10.010. PubMed PMID: 25445012; PMCID: PMC4430135.
15. Weigand MR, Pena-Gonzalez A, Shirey TB, Broeker RG, Ishaq MK, Konstantinidis KT, Raphael BH. Implications of Genome-Based Discrimination between *Clostridium botulinum*

Group I and *Clostridium sporogenes* Strains for Bacterial Taxonomy. Appl Environ Microbiol. 2015;81(16):5420-9. Epub 2015/06/07. doi: 10.1128/AEM.01159-15. PubMed PMID: 26048939; PMCID: PMC4510194.
16. Gu S, Jin R. Assembly and function of the botulinum neurotoxin progenitor complex. Curr Top Microbiol Immunol. 2013;364:21-44. Epub 2012/12/15. doi: 10.1007/978-3-642-33570-9_2. PubMed PMID: 23239347; PMCID: PMC3875173.
17. Rummel A. The long journey of botulinum neurotoxins into the synapse. Toxicon. 2015;107(Pt A):9-24. Epub 2015/09/13. doi: 10.1016/j.toxicon.2015.09.009. PubMed PMID: 26363288.
18. Inui K, Sagane Y, Miyata K, Miyashita S, Suzuki T, Shikamori Y, Ohyama T, Niwa K, Watanabe T. Toxic and nontoxic components of botulinum neurotoxin complex are evolved from a common ancestral zinc protein. Biochem Biophys Res Commun. 2012;419(3):500-4. Epub 2012/03/01. doi: 10.1016/j.bbrc.2012.02.045. PubMed PMID: 22366035.
19. Gu S, Rumpel S, Zhou J, Strotmeier J, Bigalke H, Perry K, Shoemaker CB, Rummel A, Jin R. Botulinum neurotoxin is shielded by NTNHA in an interlocked complex. Science. 2012;335(6071):977-81. Epub 2012/03/01. doi: 10.1126/science.1214270. PubMed PMID: 22363010; PMCID: PMC3545708.
20. Hill KK, Smith TJ. Genetic diversity within *Clostridium botulinum* serotypes, botulinum neurotoxin gene clusters and toxin subtypes. Curr Top Microbiol Immunol. 2013;364:1-20. Epub 2012/12/15. doi: 10.1007/978-3-642-33570-9_1. PubMed PMID: 23239346.
21. Hill KK, Xie G, Foley BT, Smith TJ, Munk AC, Bruce D, Smith LA, Brettin TS, Detter JC. Recombination and insertion events involving the botulinum neurotoxin complex genes in *Clostridium botulinum* types A, B, E and F and *Clostridium butyricum* type E strains. BMC Biol. 2009;7:66. Epub 2009/10/07. doi: 10.1186/1741-7007-7-66. PubMed PMID: 19804621; PMCID: PMC2764570.
22. Dineen SS, Bradshaw M, Johnson EA. Neurotoxin gene clusters in *Clostridium botulinum* type A strains: sequence comparison and evolutionary implications. Curr Microbiol. 2003;46(5):345-52. Epub 2003/05/07. doi: 10.1007/s00284-002-3851-1. PubMed PMID: 12732962.
23. Frankel G, Newton SM, Schoolnik GK, Stocker BA. Intragenic recombination in a flagellin gene: characterization of the H1-j gene of *Salmonella typhi*. EMBO J. 1989;8(10):3149-52. Epub 1989/10/01. PubMed PMID: 2583095; PMCID: PMC401396.
24. Harrington CS, Thomson-Carter FM, Carter PE. Evidence for recombination in the flagellin locus of *Campylobacter jejuni*: implications for the flagellin gene typing scheme. J Clin Microbiol. 1997;35(9):2386-92. Epub 1997/09/01. PubMed PMID: 9276421; PMCID: PMC229973.
25. Smith NH, Beltran P, Selander RK. Recombination of *Salmonella* phase 1 flagellin genes generates new serovars. J Bacteriol. 1990;172(5):2209-16. Epub 1990/05/01. PubMed PMID: 2129534; PMCID: PMC208845.
26. Wassenaar TM, Fry BN, van der Zeijst BA. Variation of the flagellin gene locus of *Campylobacter jejuni* by recombination and horizontal gene transfer. Microbiology. 1995;141 (Pt 1):95-101. Epub 1995/01/01. doi: 10.1099/00221287-141-1-95. PubMed PMID: 7894725.
27. Smith TJ, Hill KK, Foley BT, Detter JC, Munk AC, Bruce DC, Doggett NA, Smith LA, Marks JD, Xie G, Brettin TS. Analysis of the neurotoxin complex genes in *Clostridium botulinum* A1-A4 and B1 strains: BoNT/A3, /Ba4 and /B1 clusters are located within plasmids. PLoS One. 2007;2(12):e1271. Epub 2007/12/07. doi: 10.1371/journal.pone.0001271. PubMed PMID: 18060065; PMCID: PMC2092393.

28. Carter AT, Stringer SC, Webb MD, Peck MW. The type F6 neurotoxin gene cluster locus of group II *Clostridium botulinum* has evolved by successive disruption of two different ancestral precursors. Genome Biol Evol. 2013;5(5):1032-7. Epub 2013/05/07. doi: 10.1093/gbe/evt068. PubMed PMID: 23645598; PMCID: PMC3673618.
29. Marshall KM, Bradshaw M, Pellett S, Johnson EA. Plasmid encoded neurotoxin genes in *Clostridium botulinum* serotype A subtypes. Biochem Biophys Res Commun. 2007;361(1):49-54. Epub 2007/07/31. doi: 10.1016/j.bbrc.2007.06.166. PubMed PMID: 17658467; PMCID: PMC2346372.
30. Franciosa G, Maugliani A, Scalfaro C, Aureli P. Evidence that plasmid-borne botulinum neurotoxin type B genes are widespread among *Clostridium botulinum* serotype B strains. PLoS One. 2009;4(3):e4829. Epub 2009/03/17. doi: 10.1371/journal.pone.0004829. PubMed PMID: 19287483; PMCID: PMC2653641.
31. Hosomi K, Sakaguchi Y, Kohda T, Gotoh K, Motooka D, Nakamura S, Umeda K, Iida T, Kozaki S, Mukamoto M. Complete nucleotide sequence of a plasmid containing the botulinum neurotoxin gene in *Clostridium botulinum* type B strain 111 isolated from an infant patient in Japan. Mol Genet Genomics. 2014;289(6):1267-74. Epub 2014/08/26. doi: 10.1007/s00438-014-0887-4. PubMed PMID: 25149145.
32. Smith TJ, Hill KK, Xie G, Foley BT, Williamson CHD, Foster JT, Johnson SL, Chertkov O, Teshima H, Gibbons HS, Johnsky LA, Karavis MA, Smith LA. Genomic sequences of six botulinum neurotoxin-producing strains representing three clostridial species illustrate the mobility and diversity of botulinum neurotoxin genes. Infect Genet Evol. 2015;30:102-13. Epub 2014/12/10. doi: 10.1016/j.meegid.2014.12.002. PubMed PMID: 25489752; PMCID: PMC5459376.
33. Mansfield MJ, Adams JB, Doxey AC. Botulinum neurotoxin homologs in non-*Clostridium* species. FEBS Lett. 2015;589(3):342-8. Epub 2014/12/30. doi: 10.1016/j.febslet.2014.12.018. PubMed PMID: 25541486.
34. Wentz TG, Muruvanda T, Lomonaco S, Thirunavukkarasu N, Hoffmann M, Allard MW, Hodge DR, Pillai SP, Hammack TS, Brown EW, Sharma SK. Closed Genome Sequence of *Chryseobacterium piperi* Strain CTM^{T}/ATCC BAA-1782, a Gram-Negative Bacterium with Clostridial Neurotoxin-Like Coding Sequences. Genome Announcements. 2017;5(48). Epub 2017/12/02. doi: 10.1128/genomeA.01296-17. PubMed PMID: 29192076; PMCID: PMC5722062.
35. Zhang S, Lebreton F, Mansfield MJ, Miyashita SI, Zhang J, Schwartzman JA, Tao L, Masuyer G, Martinez-Carranza M, Stenmark P, Gilmore MS, Doxey AC, Dong M. Identification of a Botulinum Neurotoxin-like Toxin in a Commensal Strain of *Enterococcus faecium*. Cell host & microbe. 2018;23(2):169-76 e6. Epub 2018/02/06. doi: 10.1016/j.chom.2017.12.018. PubMed PMID: 29396040; PMCID: PMC5926203.
36. Doxey AC, Mansfield MJ, Montecucco C. Discovery of novel bacterial toxins by genomics and computational biology. Toxicon. 2018;147:2-12. Epub 2018/02/14. doi: 10.1016/j.toxicon.2018.02.002. PubMed PMID: 29438679.
37. Broaders E, Gahan CG, Marchesi JR. Mobile genetic elements of the human gastrointestinal tract: potential for spread of antibiotic resistance genes. Gut Microbes. 2013;4(4):271-80. Epub 2013/05/09. doi: 10.4161/gmic.24627. PubMed PMID: 23651955; PMCID: PMC3744512.
38. Marshall KM, Bradshaw M, Johnson EA. Conjugative botulinum neurotoxin-encoding plasmids in *Clostridium botulinum*. PLoS One. 2010;5(6):e11087. Epub 2010/06/17. doi: 10.1371/journal.pone.0011087. PubMed PMID: 20552020; PMCID: PMC2884020.

39. Wisniewski JA, Rood JI. The Tcp conjugation system of *Clostridium perfringens*. Plasmid. 2017;91:28-36. Epub 2017/03/14. doi: 10.1016/j.plasmid.2017.03.001. PubMed PMID: WOS:000403193700005.
40. Adams V, Li J, Wisniewski JA, Uzal FA, Moore RJ, McClane BA, Rood JI. Virulence Plasmids of Spore-Forming Bacteria. Microbiol Spectr. 2014;2(6). Epub 2015/06/25. doi: 10.1128/microbiolspec.PLAS-0024-2014. PubMed PMID: 26104459.
41. Heap JT, Pennington OJ, Cartman ST, Carter GP, Minton NP. The ClosTron: a universal gene knock-out system for the genus *Clostridium*. J Microbiol Methods. 2007;70(3):452-64. Epub 2007/07/31. doi: 10.1016/j.mimet.2007.05.021. PubMed PMID: 17658189.
42. Lambowitz AM, Zimmerly S. Mobile group II introns. Annu Rev Genet. 2004;38:1-35. Epub 2004/12/01. doi: 10.1146/annurev.genet.38.072902.091600. PubMed PMID: 15568970.
43. Kuehne SA, Heap JT, Cooksley CM, Cartman ST, Minton NP. ClosTron-Mediated Engineering of *Clostridium*. In: Williams JA, editor. Strain Engineering: Methods and Protocols: Humana Press; 2011. p. 389-407.
44. Adams V, Bantwal R, Stevenson L, Cheung JK, Awad MM, Nicholson J, Carter GP, Mackin KE, Rood JI, Lyras D. Utility of the clostridial site-specific recombinase TnpX to clone toxic-product-encoding genes and selectively remove genomic DNA fragments. Appl Environ Microbiol. 2014;80(12):3597-603. Epub 2014/04/01. doi: 10.1128/AEM.04285-13. PubMed PMID: 24682304; PMCID: PMC4054144.
45. Al-Hinai MA, Fast AG, Papoutsakis ET. Novel system for efficient isolation of *Clostridium* double-crossover allelic exchange mutants enabling markerless chromosomal gene deletions and DNA integration. Appl Environ Microbiol. 2012;78(22):8112-21. Epub 2012/09/18. doi: 10.1128/AEM.02214-12. PubMed PMID: 22983967; PMCID: PMC3485963.
46. Ng YK, Ehsaan M, Philip S, Collery MM, Janoir C, Collignon A, Cartman ST, Minton NP. Expanding the repertoire of gene tools for precise manipulation of the *Clostridium difficile* genome: allelic exchange using *pyrE* alleles. PLoS One. 2013;8(2):e56051. Epub 2013/02/14. doi: 10.1371/journal.pone.0056051. PubMed PMID: 23405251; PMCID: PMC3566075.
47. Dong H, Tao W, Gong F, Li Y, Zhang Y. A functional *recT* gene for recombineering of *Clostridium*. J Biotechnol. 2014;173:65-7. Epub 2014/01/05. doi: 10.1016/j.jbiotec.2013.12.011. PubMed PMID: 24384234.
48. Pyne ME, Moo-Young M, Chung DA, Chou CP. Coupling the CRISPR/Cas9 System with Lambda Red Recombineering Enables Simplified Chromosomal Gene Replacement in *Escherichia coli*. Appl Environ Microbiol. 2015;81(15):5103-14. Epub 2015/05/24. doi: 10.1128/AEM.01248-15. PubMed PMID: 26002895; PMCID: PMC4495200.
49. van Pijkeren JP, Britton RA. Precision genome engineering in lactic acid bacteria. Microb Cell Fact. 2014;13 Suppl 1(Suppl 1):S10. Epub 2014/09/05. doi: 10.1186/1475-2859-13-S1-S10. PubMed PMID: 25185700; PMCID: PMC4155826.
50. Wang Y, Zhang ZT, Seo SO, Choi K, Lu T, Jin YS, Blaschek HP. Markerless chromosomal gene deletion in *Clostridium beijerinckii* using CRISPR/Cas9 system. J Biotechnol. 2015;200:1-5. Epub 2015/02/15. doi: 10.1016/j.jbiotec.2015.02.005. PubMed PMID: 25680931.
51. Xu T, Li YC, Shi Z, Hemme CL, Li Y, Zhu YH, Van Nostrand JD, He ZL, Zhou JZ. Efficient Genome Editing in *Clostridium cellulolyticum* via CRISPR-Cas9 Nickase. Applied and Environmental Microbiology. 2015;81(13):4423-31. Epub 2015/04/26. doi: 10.1128/Aem.00873-15. PubMed PMID: WOS:000355844800020; PMCID: PMC4475897.

Literature Review: A brief overview of plasmids in *C. botulinum* and related species

Abstract

Plasmids are widespread in *Clostridium botulinum* and related neurotoxigenic species. Many *C. botulinum* isolates contain large plasmids ranging in size from 50 - 300 kb, with several of these plasmids encoding botulinum neurotoxin (BoNT) genes. Recent research suggests that at least some of these large plasmids are mobilizable elements that can transfer to other clostridial species by conjugation. Aside from the neurotoxins, however, the genomic content of these extrachromosomal elements is understudied. The plasmid content of sequenced *C. botulinum* strains could contain clues to the evolutionary history of the species, and the vast number of hypothetical proteins found on *C. botulinum* plasmids could serve as untapped sources of genetic tools in the clostridia. Importantly, a characterization of the mobility of *C. botulinum* toxin plasmids may help direct surveillance and safety measures for the prevention of botulism. A short overview of *C. botulinum* plasmids and comments on their relevance to these topics are presented in this review chapter.

Introduction

While extrachromosomal elements like plasmids are widespread in bacteria and other domains, their evolutionary persistence is often unexplained (1). The maintenance of plasmids as independent replicons, and their failure to be lost to segregation or to integrate beneficial genes into the host chromosome, has been termed the "plasmid paradox" (2). One model in which plasmids are predicted to persist in a population is by their carriage of genes that provide local adaptations, or advantages under sporadic or stochastic conditions (3). Virulence factors, which may benefit a cell specifically during pathogenicity, are considered a local adaptation (3).

Many notable pathogens, such as *Bordetella pertussis* and *Staphylococcus aureus*, encode protein toxins that facilitate infection by establishing a niche or evading host immune response (4). Other bacteria, namely the clostridia, maintain toxin genes whose fitness implications are less evident. Unlike the enteric pathogens, for example, whose virulence plasmids are the subject of a thorough recent review (5), clostridia encode neurotoxins whose evolutionary advantage is not clear. The environment in which clostridial neurotoxins give strains a competitive advantage has not been truly elucidated. *C. botulinum* colonizes the intestinal tract in rare instances, but the specificity with which BoNT targets neurons suggests that the toxin does not promote infection or invasion. Indeed, the toxin and its host strain often seem uncoupled from one another.

Botulinum neurotoxins are found in gene clusters flanked by partial IS elements, and may be encoded on the chromosome, on phages, or on plasmids. As noted by Brüggemann, the presence of clostridial toxins on extrachromosomal elements (e.g. plasmid-borne tetanus toxin and the pathogenicity locus of *C. difficile*) suggests that they are or were once part of the flexible gene pool, conferring increased fitness or pathogenicity (6). By examining the genomics and evolution of toxin-encoding plasmids in clostridia, we may be able to glean insights into the

unique role of BoNTs in the genus. In this review, we seek to characterize the current set of *C. botulinum* plasmids and mobile genetic elements, particularly those that encode botulinum neurotoxin. Although the evolutionary significance of BoNTs remains enigmatic, we believe that a focus on the carriage and transmissibility of plasmid-borne toxins will help elucidate the environmental, genetic, and physiological properties of *C. botulinum* as a whole.

Clostridium botulinum and its neurotoxins

The diverse *Clostridium botulinum* strains expressing the various BoNT serotypes are classified into four physiological Groups, designated with numerals I-IV. Group I *C. botulinum* strains are termed proteolytic, as they encode extracellular proteases for acquiring amino acid nutrients and for nicking the single chain BoNT molecule; these strains have an optimum growth temperature of 37°C and can produce BoNTs of serotypes A, B, and F (7). Group II strains are termed nonproteolytic: they do not encode their own nicking protease, but rather rely on proteolytic enzymes in the environment to convert their neurotoxins to the dichain form. They prefer lower temperatures (30°C) and higher water activity for growth and toxin formation, and they may produce BoNTs of serotypes B, E, and F (7).

Group III *C. botulinum* strains produce BoNT/C, BoNT/D, or chimeric toxins (BoNT/CD or BoNT/DC) that appear to be hybrids of the two serotypes. These serotypes are encoded on large prophages that propagate extrachromosomally; they cause avian and nonhuman mammalian botulism (8). Finally, Group IV strains produce BoNT/G, which is typically plasmid-encoded and has been implicated in rare human intoxications (9).

Historically, BoNT production was the sole criterion required for designation of the species *C. botulinum*, a framework that has resulted in the complicated phylogeny of the species today. The availability of sequence information has not only revealed the great genetic distance between the four Groups, but also has enabled the characterization of BoNT-producing strains of other clostridial species, such as *C. sporogenes*, *C. butyricum*, and *C. baratii*.

The *C. botulinum* plasmidome

Before the widespread availability of genome sequencing, the locus of *bont* genes was probed imprecisely. It was thought that Group IV *C. botulinum*, the source of *bont/g* and the

closest relative of *C. tetani*, was the sole clade that produced BoNT from a plasmid (9, 10). Small plasmids from other Groups were identified, but the strains that carried them were still toxigenic upon plasmid curing (11). In the last decade, however, plasmids in all Groups of *C. botulinum* have been shown to encode *bont* genes (8, 12-15). Furthermore, the *bont/g* locus has been assigned to the chromosome in at least one Group IV strain (16). These developments, though they have complicated the traditional understanding of *bont* loci, provide enticing new avenues of research. Beyond the ability of some plasmids to carry *bont* genes, little is known about their functionality in *C. botulinum*. To better characterize the plasmidome of *C. botulinum*, we downloaded the collection of all available closed *C. botulinum* plasmid sequences (Figure 1a) from NCBI and analyzed it against the clusters of orthologous groups (COGs) database (17). The most abundant COGs present are those with roles in replication, recombination, and repair, possibly representing the many phage-associated proteins that have integrated into *C. botulinum*'s extrachromosomal elements (Figure 1b).

Other accessory functions present on *C. botulinum* plasmids include central dogma processes (e.g. transcription and translation) and transport and metabolism of macromolecules (Figure 1b). While we have not correlated these plasmid COGs with particular bacterial phenotypes, they suggest that *C. botulinum* is poised to adapt to various physiological conditions. For example, plasmid gene products are predicted to include superoxide dismutases (COG class P) and multidrug transport systems (COG class V), which could help bacteria respond to environmental stressors (Figure 1b). Horizontal gene transfer of these plasmid genes could create *C. botulinum* strains with novel resistance profiles. On the other hand, plasmids incur a metabolic cost to the host, and the accumulation of cell-wall-associated proteins (including conjugative transfer pili) may generate greater susceptibility to bacteriophage infection (18-20).

The extrachromosomal gene pool may therefore offer tools for genetic engineering of strains and targets for controlling their spread.

Plasmids tend to be distinguished from chromosomes or the intermediately sized "chromids" by their lack of core or essential genes (21, 22). While we have not undertaken extensive research into the essentiality of the plasmid genes described here, it should be noted that none of the genomes analyzed contain genes for rRNA. A minority of *C. botulinum* plasmid genomes (17 of 80 in this dataset) have annotations suggesting they encode tRNA genes, though the maximum number of plasmid-borne tRNAs in this dataset is two. These data—and the evidence that plasmid curing has been performed experimentally in neurotoxigenic clostridia (11, 23)—suggest that the plasmids of *C. botulinum* do not represent chromids or second chromosomes.

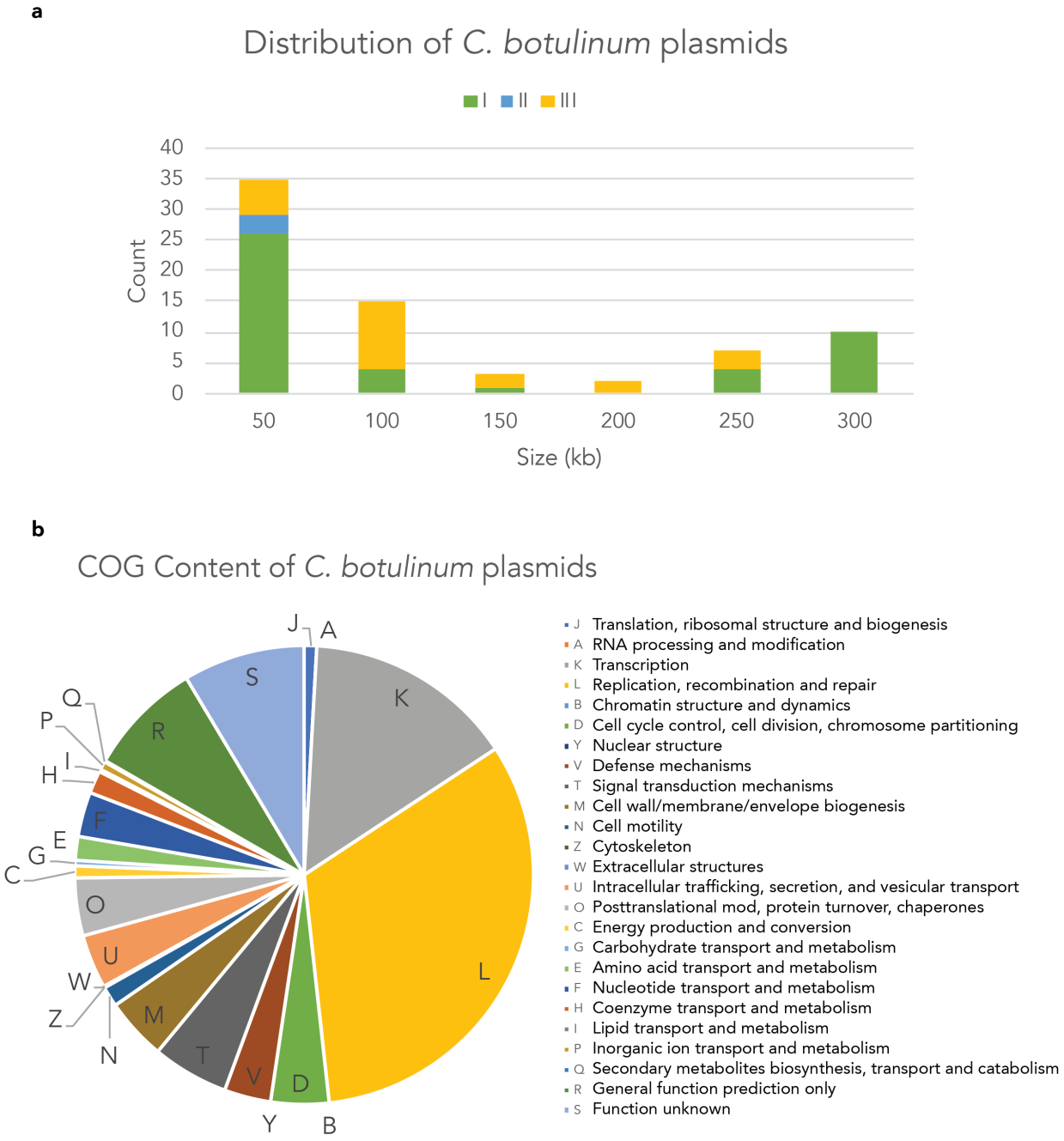

Figure 1. Many small nontoxigenic plasmids exist in the pangenome of *C. botulinum*. Annotated plasmid sequences were downloaded from the NCBI Genome page for *Clostridium botulinum* (https://www.ncbi.nlm.nih.gov/genome/plasmids/726). (a) Plasmids were assigned to a physiological Group of *C. botulinum* based on their Clade ID and sorted by size in kilobasepairs. (b) Protein sequences from the plasmids were assigned to COG families using the WebMGA server (24). Note that sequences in contigs and scaffolds are not included in this analysis.

Plasmid-borne clostridial neurotoxins

C. botulinum genomic analyses are generally driven by a search for botulinum neurotoxin (*bont*) genes. The identification of plasmid-borne *bont* loci throughout the four physiological Groups of *C. botulinum* and in other clostridia is reviewed below and collected in Table S1. Comments on the size and selected features of these toxin plasmids are also included in the text.

Group I C. botulinum

The vast differences in botulinum neurotoxin expression across *C. botulinum* strains have long been a curiosity in the field. Shortly after BoNT serotypes were divided into subtypes, research sought a correlation between the genetic locus of the neurotoxin and its protein expression levels. Strains that produced "limited and inconsistent" quantities of BoNT (12) were chosen for analysis by pulsed-field gel electrophoresis (PFGE) to assess whether their neurotoxin loci differed. Surprisingly, probes for the neurotoxin genes in these strains (*bont/a* in the A3 strain Loch Maree and *bont/a* and */b* in the bivalent strain 657Ba) hybridized to large extrachromosomal elements (12). This was the first evidence of plasmid-borne neurotoxins in Group I *C. botulinum*.

The genomic loci of these and other Group I neurotoxins were quickly confirmed by sequence data. Smith et al. showed that the aforementioned Loch Maree and 657Ba strains, producing BoNT subtypes /A3, /A4, and /B5 (formerly known as "bivalent B"), encoded these neurotoxins on large plasmids (25). pCLK, carrying BoNT/A3, is approximately 267 kilobasepairs, while pCLJ, carrying both /A4 and /B5, is slightly larger at 270 kb. A third strain sequenced in this study, the BoNT/B1-producing *C. botulinum* Okra, encodes its neurotoxin on a smaller plasmid, the ~149 kb pCLD (25).

Surveys of neurotoxin loci have not been comprehensive, but the evidence of plasmid-borne toxins in Group I *C. botulinum* continues to accumulate. Franciosa et al. found that the *bont/b* gene was located on a plasmid in more than half of the strains tested (26). The frequency of plasmid localization varied by BoNT/B subtype, and the plasmids varied in size from 55 kb to 245 kb (26). Dover et al. added to the corpus of Group I neurotoxin plasmids upon sequencing *C. botulinum* Af84, finding *bont/f5* on a 246 kb plasmid (27), and Raphael et al. extended this observation to additional *bont/f5* strains (28).

C. botulinum plasmids harboring *bont/b* genes can be lost upon serial passage, indicating that the toxigenicity in Group I can be unstable (29). Hosomi et al. also demonstrated plasmid instability in the BoNT/B strain 111; the *bont/b2*-encoding plasmid pCB111 was very unstable, perhaps due to the absence of a death-on-curing protein that is present in other clostridial plasmids (30). Increased surveillance and sequencing of Group I *C. botulinum* strains is certainly necessary, as plasmid-borne toxins have been implicated in cases of infant botulism and wound botulism (31-33).

Group II C. botulinum

As with most *C. botulinum* Groups, the plasmid content of nonproteolytic Group II strains was investigated long before the toxin loci were known. Strom et al. isolated a variety of plasmids from 44 *C. botulinum* strains (34). The strains chosen encompassed all BoNT serotypes; however, no particular phenotypes were ascribed to the plasmids themselves (34). This work was prescient, however, as it postulated that the presence of a similarly-sized plasmid in strains of wide geographical origin could be evidence of a shared plasmid-borne toxin (34). Franciosa et al. (2009) and Hill et al. (2009) later confirmed via PFGE and whole-genome

sequencing, respectively, that the non-proteolytic subtype of BoNT/B could be carried on a plasmid (26, 35).

Neurotoxin plasmids in Group II *C. botulinum* are, on the whole, smaller than those in Group I. A survey of non-proteolytic BoNT/B strains found that 23 of 26 strains carried the *bont* gene on plasmids, and the remaining three strains seemed to have lost their toxigenicity, as no hybridization signals were detected on their chromosomes (14). Plasmids in this survey ranged from 47 to 63 kb and demonstrated a strong correlation between plasmid lineage and geographical origin (14).

In contrast, the type E strains of Group II *C. botulinum* rarely encode their neurotoxins on plasmids. PFGE analysis of 36 type E strains indicated that only three contained the *bont/e1* gene on a plasmid (13). Genetic analysis extended this observation, as only 6% of 150 type E genomes surveyed had plasmid-borne neurotoxins (36).

Group III C. botulinum

Historically, less is known about Group III *C. botulinum*, whose BoNT/C and /D neurotoxins are responsible for animal botulism. These toxins are known to be recombinogenic, as chimeric variants (BoNT/CD and /DC) are common in nature (37). Group III toxins are carried on an unstable bacteriophage that is genetically conserved to approximately the same degree as the chromosome (8).

A number of epidemiological studies investigating the plasmid carriage of Group III outbreak strains have been performed. Animal botulism is an emerging concern, particularly in poultry production in Europe (38). In cases of botulism caused by BoNT/CD strains, correlations can be found between the presence of the neurotoxin and other markers such as alpha-toxin and C2, suggesting that there may be non-BoNT elements that are important for

pathogenicity (38). The same study showed that mobile elements from Group III *C. botulinum* are found even in *bont*-negative samples, indicating that the phage responsible for toxigenicity can be lost during an outbreak (38).

Aside from the neurotoxin bacteriophage, Group III *C. botulinum* strains carry several plasmid lineages. An early sequencing study found that 13.5% of the total genomic content in Group III *C. botulinum* was plasmid DNA (8). The largest extrachromosomal element carried the neurotoxin, while the smaller plasmids carried a variety of other toxins and resistance genes. These non-BoNT plasmids in Group III are thought to contain a greater number of mobile elements and undergo faster genetic drift (8).

Group IV C. botulinum

Eklund et al. were the first to correlate the presence of a plasmid with neurotoxin production in *C. botulinum* (10). An 81 megadalton plasmid carried by six toxigenic isolates of type G *C. botulinum* could be cured by consecutive daily transfers at 44°C; when this plasmid was lost, the strains no longer produced BoNT/G (10). Once the sequence of *bont/g* was determined (39), Zhou et al. created probes based on the gene and showed that they hybridized to plasmid, but not chromosomal, DNA in strains 117 and 89 (9). Zhou et al. also confirmed that two genes in the *bont/g* cluster, HA and NTNH, were linked to the plasmid-borne neurotoxin (9). This study further refined the estimate of the type G plasmid's size to approximately 114 kilobasepairs (9).

Two of the strains discussed in Eklund et al. 1988 were isolated from Argentina and were the subject of Zhou et al.'s *bont/g* hybridization study (9, 10). The four remaining strains in the Eklund study were isolated from autopsy specimens in Switzerland by Sonnabend et al. (40). Although the lethality of BoNT/G was shown in monkeys, chickens, and guinea pigs, outbreaks

of botulism caused by this toxin have not been reported (41). Type G strains seem to produce comparatively low levels of toxin and do not readily sporulate, making detection more difficult (42). Halpin et al. recently reported a complete plasmid sequence from *C. argentinense* strain 89G on which the *bont/g* gene was present (15). Much like other BoNT-encoding plasmids, pRSJ17_1 is relatively large (140 kb) and poorly annotated, containing many hypothetical proteins (GenBank CP014175).

Clostridium butyricum

Shortly after the *C. butyricum* neurotoxin gene was identified as *bont/e*, this locus was shown to be plasmid-borne (43). Molecular studies of the *C. butyricum* neurotoxin elements initially proposed that they were linear plasmids, although this was subsequently revised to circular megaplasmids (44). Demonstrating the size and toxigenicity of these megaplasmids, a *C. butyricum* strain producing BoNT/E was isolated from a case of infant botulism and subject to whole genome sequencing. The *bont/e4* gene in this *C. butyricum* isolate was located on a plasmid of approximately 820 kb (45).

The various megaplasmids of neurotoxgenic *C. butyricum* share structural, metabolic, and regulatory genes (44). Larger megaplasmids from this group carry an interesting CRISPR-Cas element whose spacers have homology to phage and plasmid sequences from clostridia and other intestinal bacteria. The authors suggest that the megaplasmids might therefore be important for resisting invasion by genetic elements and establishing residence in the human or animal environment (44). The *C. butyricum* megaplasmids also contain numerous regulatory elements that influence toxin expression and have pleiotropic effects on bacterial growth and adaptation (23).

Clostridium baratii

Following the diagnosis of infant botulism cases caused by *Clostridium baratii*, efforts were undertaken to compare the active neurotoxin in *C. baratii* to its relatives in *C. botulinum*. Gimenez et al. found that the so-called "baratii neurotoxin" was similar in many respects to the characterized botulinum neurotoxin serotypes, and postulated that this result would be valuable in addressing "questions regarding the location of the neurotoxin gene and its exchange or acquisition by the nonneurotoxic *C. baratii* and toxigenic *C. botulinum*" (46). The baratii neurotoxin was later recategorized as BoNT/F, but the same questions remain as to its movement into *C. baratii*. As toxigenic *C. baratii* can carry *bont/f7* on plasmids, it is possible that the neurotoxin gene was obtained by horizontal gene transfer (45).

Mobility of BoNT plasmids

The widespread availability of whole genome sequencing has led to the identification of numerous extrachromosomal elements in *C. botulinum* and related species. It has also presented the opportunity to compare *bont* genes across various replicons. As the clostridia are a genomically diverse genus of bacteria, horizontal gene transfer is a probable explanation for the dissemination of *bont* genes between otherwise distantly related strains. Furthermore, because *bont* gene clusters are flanked by partial IS elements, it has long been assumed that the mode of transfer is by transposon (or by bacteriophage in the case of Group III *C. botulinum*). However, the widespread presence of *bont* gene clusters on plasmids raises questions as to whether plasmid conjugation also provides a means of horizontal *bont* gene transfer, even if the *bont*-bearing plasmids originate from initial transposon insertion events. Direct and indirect lines of evidence for the mobility of plasmids encoding BoNTs are summarized below.

Experimental evidence of bont *gene transmission*

Transmissibility of the Group I plasmids pCLJ and pCLK and the Group II plasmid pCLL into selected Group I C. *botulinum* recipients was first demonstrated by Marshall et al. (47). The host range of pCLJ, the dual-BoNT-encoding plasmid of *C. botulinum* strain 657Ba, was subsequently extended to additional clostridial genomic lineages, including *C. sporogenes* and *C. butyricum* (48). *C. sporogenes* transconjugants can express active BoNT/A4 from the pCLJ locus, indicating that these strains may become toxigenic upon plasmid acquisition (48).

In Group III *C. botulinum*, the fluidity of plasmids and bacteriophages can also convert diverse strains to toxigenicity (49). Horizontal gene transfer is an active and ongoing process in Group III, and the resulting strains are genetically very complex (49). Group III's extrachromosomal elements are useful in defining the overall genomic content of the bacteria—isolates can be categorized into at least eight phage groups based on the presence or absence of certain replicons—but this classification compares poorly to a SNP-based core phylogeny when it comes to identifying the lineage of a particular outbreak strain (37). Understanding the mobility of *bont* genes and their associated bacteriophages and plasmids in Group III is generally more informative at the population level, rather than strain by strain.

Evidence supporting plasmid mobility in *C. butyricum* was first given by Hauser et al., who noted that BoNT/E plasmids in toxigenic *C. butyricum* isolates were absent from nontoxigenic *C. butyricum* and related strains (43). Although persuasive, this observation revealed little about the directionality or dynamics of gene transfer. Having found that the *bont/e* sequence in toxigenic *C. butyricum* was similar to that in *C. botulinum*, Fujii et al. proposed that chromosomal DNA containing the toxin gene might be transferred between the two organisms, e.g. by bacteriophage (50). Zhou et al. investigated this possibility and determined that transduction of the toxin gene from *C. butyricum* to a non-toxigenic *C. botulinum* type E strain

was only permitted when a defective phage was made infective by a "helper strain" (51). Although the molecular mechanisms await elucidation, it is apparent that plasmid mobility has played a role in shaping the modern phylogeny of neurotoxigenic clostridia.

Genomic evidence of bont *gene mobility*

At present, the precise mechanism of plasmid transfer is not clear, and no recombination of *bont* genes following mobilization to new strains has been experimentally observed. Nonetheless, whole genome analyses of various *C. botulinum* strains indicate that *bont* gene clusters are subject to horizontal gene transfer and may move between plasmid and chromosomal loci. At least one Group I *C. botulinum* isolate exhibits a chromosomal *bont/b* gene that is flanked by a "pCLJ-like" region, and at least one Group IV *C. botulinum* strain encodes the traditionally plasmid-borne BoNT/G from its chromosome (15, 52).

Circumstantial evidence for *bont* gene transfer is also seen in the various strains of the *C. sporogenes* lineage that produce BoNT/B from a plasmid (33, 53, 54). *C. sporogenes* is the traditional nontoxigenic surrogate for Group I *C. botulinum*. Certain *C. sporogenes* strains, however, encode BoNT/B2 and have sequence similarity with the *C. botulinum* neurotoxin plasmid pCLD (54). It is likely that the *C. sporogenes* strains acquired their toxigenicity via horizontal gene transfer from *C. botulinum*, and thus that the boundary between these two species is not firm.

Early in the study of *C. botulinum* plasmids, Eklund et al. reported that six independent Group IV strains producing BoNT/G had similar genomic banding patterns by restriction digest but differing phenotypes with respect to sporulation and plasmid stability (10). As the Group IV strains examined carry the same size plasmid despite their geographic and phenotypic divergence, it is possible that the plasmid is transmissible or that maintenance of the toxin

plasmid is under positive selection (10). Like the BoNT/G plasmids, BoNT/F5 plasmids are also probable candidates for mobilization, as similar plasmid sequences are present in diverse genomic backgrounds of Group I *C. botulinum* (28).

More tellingly, the Group II *C. botulinum* neurotoxin plasmid sequences themselves reveal substantial evidence of recombination and mobility. Three BoNT/B plasmid lineages were identified by Carter et al. (2014), with one classified as a hybrid between the other two (14). Group II strains with BoNT/E plasmids each carry a 24 kb cassette, which contains the neurotoxin cluster and flanking genes, that has split a plasmid-borne helicase and inserted therein (36). This evidence and the incredible successive recombination exhibited at the *bont/f6* locus suggest that exchange between plasmid and chromosomal replicons could be more active in Group II *C. botulinum* (55)—although a genomic comparison easily separates Group II *C. botulinum* strains into three clades that mostly parallel their toxin serotype, which may imply reduced mobility of the *bont* gene clusters (56).

Carter et al. provide sequence data supporting plasmid transmission in Group II *C. botulinum*, noting that all BoNT/B4 plasmids in their 2014 survey and all BoNT/E plasmids in their 2016 study carried genes associated with conjugation (14, 36). Conversely, the mobility of plasmids into Group II *C. botulinum* may be restricted, as BoNT/E plasmids apparently possess a CRISPR locus with spacers targeting other Group II plasmids (36). As is the case in their Group II *C. botulinum* counterparts, *C. butyricum* strains with plasmid-borne *bont/e* genes also encode CRISPR spacer sequences targeting foreign Group II *C. botulinum* plasmid DNA (44). The dueling forces of plasmid conjugation and defense against foreign DNA present a compelling area of research in *C. botulinum*.

Finally, and most surprisingly, an active BoNT-like toxin has been identified on a conjugative plasmid in *Enterococcus faecium* (57). This putative toxin, currently known as

BoNT/En, is found as part of an *orfX* cluster on a ~207 kb plasmid (57). The plasmid (pBoNT/En) belongs to the repUS15 family; its sequence suggests that it encodes proteases, bacteriocins, and a type IV secretion system in addition to the neurotoxin (57). Plasmids of this family are known to be transmissible, as evidenced by the circulation of vancomycin resistance among *Enterococcus* strains (58).

Virulence plasmids

The toxin plasmids of *C. botulinum* are apparently nonessential for the survival of the organism, but some can be mobilized between strains. Should they be considered virulence plasmids? This classification is apt, so long as botulinum neurotoxin is considered a virulence factor. Pilla and Tang describe a prototypical enteropathogenic virulence plasmid as being greater than 40 kb in size, possessing toxin-antitoxin and partitioning systems, and encoding "virulence genes," including toxins (5). The functions attributed to virulence genes, though, give their host bacteria clear advantages in invasion, adhesion, colonization, resistance to host defenses, etc. (5, 59). *C. botulinum* spores may germinate in anaerobic conditions in the vertebrate intestinal tract, but BoNT itself does not seem to aid in this pathology (60). It is perhaps more useful to frame the toxin as a means of disseminating spores throughout the environment, as can occur when cadavers decompose following death by animal botulism (61). The potential to disperse genes to new and varied hosts is an advantage at the population scale and could help explain the persistence of plasmids that might otherwise have fitness costs (62, 63). Benoit makes the alternative case that *bont* is a "selfish gene," diversifying over time without providing a selective advantage to its host (64). While the evolutionary arguments are not settled, there is precedent for classifying BoNTs as virulence factors (65) and thus classifying the extrachromosomal BoNT elements as virulence plasmids (66). It seems reasonable to allow

this naming convention to stand, even if the particular circumstances in which BoNT plasmids increase the fitness of clostridia are not obvious.

Conclusions

Underlying our research into the BoNT plasmids of *C. botulinum* is the possibility that the toxins may be mobilized to diverse strains. But how likely is this? BoNTs and homologous protein sequences are found in species outside of *C. botulinum*, but outbreaks of botulism from these sources are rare (67). The challenges inherent in classification and expression of non-botulinum toxins have revived debates about the definition of a BoNT. Should it hinge on biological activity, sequence similarity, or some combination of factors? Much as the nomenclature of BoNT subtypes has been workshopped and formalized (68), the discovery of BoNT-like proteins outside of the traditional *C. botulinum* species groupings will necessitate a reevaluation of these toxins.

What governs the mobility of *C. botulinum* plasmids? As the mechanism of plasmid replication has not been well-studied, their segregation and compatibility are not known. It is possible that bacterial DNA transfer in the natural environment is prevented by the incompatibility of plasmid replicons or by active exclusion, e.g. by CRISPR-Cas systems. Negahdaripour et al. have performed sequence analysis of the spacers in *C. botulinum* CRISPR arrays and found hits to bacteriophages, prophages, and plasmids (69). These CRISPR-Cas systems have not been experimentally verified, but they likely serve as one mechanism by which plasmid transfer is restricted within the clostridia.

Still, the potential for DNA exchange remains. Plasmids that are hybrids of multiple lineages exist in *C. botulinum* Group I (70) and Group II (14), and the BoNTs themselves are chimeric in Group III. Even if the evolutionary recombination events occurred in the distant

past, as is often suggested by the divergence of strains, plasmid mobility is a valuable area of study.

In this review, we have endeavored to catalog the neurotoxin-encoding plasmids of *Clostridium botulinum* and related species. To our knowledge, the last survey of *C. botulinum* plasmids across the physiological Groups was made some thirty years ago (34), before the advent of widespread genome sequencing. Since then, the botulism community has made great strides in identifying and characterizing various *bont* genes and the neurotoxins they specify. Still, more attention should be paid to the plasmid and chromosomal contexts of these genes if their evolutionary role is to be elucidated. The distribution and mobility of plasmids, and their genetic makeup beyond the neurotoxin clusters, are crucial to understanding the health and safety threats posed by botulism.

Table S1. Extrachromosomal *bont* loci in *C. botulinum* and related species.

Species	Group	Strain	Serotype	Accession Number	Reference
C. botulinum	I	Loch Maree	A	NC_010418.1	(25)
C. botulinum	I	CDC 1436	A, B	NZ_CP006909.1	(16)
C. botulinum	I	A2B3 87	A, B	NZ_AUZB01000012.1	(71)
C. botulinum	I	A2B7 92	A, B	NZ_AUZA01000014.1	(71)
C. botulinum	I	AM1195	A, B	CP013700.1, LFPH00000000	(53)
C. botulinum	I	CDC_67071	B	NZ_CP013241.1	
C. botulinum	I	Prevot 594	B	NZ_CP006901.1	(16)
C. botulinum	I	B2 433	B	NZ_AUYZ01000011.1	(71)
C. botulinum	I	111	B	NC_025146.1	(30)
C. botulinum	I	Okra	B	NC_010379.1	(25)
C. botulinum	I	AM553	B	LFPK00000000	(53)
C. botulinum	I	AM370	B	LFPJ00000000	(53)
C. botulinum	I	AM1195	B	LFPH00000000	(53)
C. botulinum	I	CDC 66221	B	LAGO01000000	(54)
C. botulinum	I	B2 450	B	JXSU00000000	(33)
C. botulinum	I	Osaka05	B	BAUF00000000	(72)
C. botulinum	I	CDC-555	B		(26)
C. botulinum	I	CDC-620	B		(26)
C. botulinum	I	CDC-628	B		(26)
C. botulinum	I	CDC-668	B		(26)
C. botulinum	I	CDC-1588	B		(26)
C. botulinum	I	CDC-1758	B		(26)
C. botulinum	I	CDC-1852	B		(26)
C. botulinum	I	CDC-1872	B		(26)
C. botulinum	I	CDC-2064	B		(26)
C. botulinum	I	CDC-2094	B		(26)
C. botulinum	I	CDC-2113	B		(26)
C. botulinum	I	CDC-2292	B		(26)
C. botulinum	I	CDC-2306	B		(26)
C. botulinum	I	CDC-2329	B		(26)
C. botulinum	I	CDC-2358	B		(26)
C. botulinum	I	CDC-2589	B		(26)
C. botulinum	I	CDC-2593	B		(26)
C. botulinum	I	CDC-2746	B		(26)
C. botulinum	I	CDC-2978	B		(26)
C. botulinum	I	CDC-5078	B		(26)
C. botulinum	I	CDC-5153	B		(26)
C. botulinum	I	CDC-5168	B		(26)
C. botulinum	I	CDC-5250	B		(26)
C. botulinum	I	CDC-5323	B		(26)
C. botulinum	I	CDC-7699	B		(26)
C. botulinum	I	MDb02	B		(26)
C. botulinum	I	ISS-333	B		(26)
C. botulinum	I	Okayama2011	B		(70)
C. botulinum	I	NSW4_B6	B		(32)

C. botulinum	I	657Ba	B, A	NC_012654.1	(25)
C. botulinum	I	ISS-87	B, A		(26)
C. botulinum	I	ISS-92	B, A		(26)
C. botulinum	I	10258	B, F	LFON00000000	(53)
C. botulinum	I	(Bf)	B, F	ABDP01000001.1	(35)
C. botulinum	I	Af84	F	NZ_AOSX01000021.1	(27)
C. botulinum	I	BrDura	F	CP014152.1	(73)
C. botulinum	I	CDC 54074	F		(28)
C. botulinum	I	CDC 54075	F		(28)
C. botulinum	I	CDC 54079	F		(28)
C. botulinum	I	CDC 54084	F		(28)
C. botulinum	I	CDC 54085	F		(28)
C. botulinum	I	CDC 54090	F		(28)
C. botulinum	I	CDC 54096	F		(28)
C. botulinum	II	Eklund 17B	B	NC_018653.1, NC_010680.1	(74)
C. botulinum	II	DB2	B	KJ776585	(14)
C. botulinum	II	KapchunkaB8	B	KJ776584	(14)
C. botulinum	II	KapchunkaB3	B	KJ776583	(14)
C. botulinum	II	CDC3897	B	KJ776582	(14)
C. botulinum	II	IFR_05/025	B	KJ776581	(14)
C. botulinum	II	CDC5900	B	KJ776580	(14)
C. botulinum	II	KapchunkaB2	B	KJ776579	(14)
C. botulinum	II	CDC3875	B	KJ776578	(14)
C. botulinum	II	Colworth BL151	B	KJ776577	(14)
C. botulinum	II	Eklund 2B	B	KJ776576	(14)
C. botulinum	II	CDC-706	B		(26)
C. botulinum	II	CDC-4848	B		(26)
C. botulinum	II	CB11/1-1	E	NZ_AORM00000000.1	(13, 36)
C. botulinum	II	FI1111E1	E	KT897280	(36)
C. botulinum	II	SWKR38E2	E	KT897279	(36)
C. botulinum	II	FWSKR40E1	E	KT897278	(36)
C. botulinum	II	ST0210E1	E	KT897277	(36)
C. botulinum	II	INGR16-02E1	E	KT897276	(36)
C. botulinum	II	IFR 12/29	E	KT897275	(36)
C. botulinum	II	K51	E	AM695761	(13)
C. botulinum	II	K22	E		(13)
C. botulinum	III	C-Stockholm	C	AESA00000000.2	(49)
C. botulinum	III	CIP 4165	C		(38)
C. botulinum	III	NRCB5	C		(38)
C. botulinum	III	7573/3/12	C		(38)
C. botulinum	III	1272	C		(38)
C. botulinum	III	CP07	C		(38)
C. botulinum	III	Lan1	C		(38)
C. botulinum	III	Lan2	C		(38)
C. botulinum	III	Bal64	C		(38)
C. botulinum	III	1663	C		(38)
C. botulinum	III	2883	C		(38)

C. botulinum	III	850131	C		(38)
C. botulinum	III	468	C		(38)
C. botulinum	III	CKIII	C		(38)
C. botulinum	III	15586	C		(38)
C. botulinum	III	870505	C		(38)
C. botulinum	III	75965	C		(38)
C. botulinum	III	RKI-1	C		(38)
C. botulinum	III	RKI-2	C		(38)
C. botulinum	III	71840	CD	SRS1009734	(37, 38)
C. botulinum	III	69285	CD	SRS1009733	(37, 38)
C. botulinum	III	58752	CD	SRS1009732	(37, 38)
C. botulinum	III	58272	CD	SRS1009731	(37, 38)
C. botulinum	III	55741	CD	SRS1009730	(37, 38)
C. botulinum	III	50867	CD	SRS1009716	(37, 38)
C. botulinum	III	49511	CD	SRS1009715	(37, 38)
C. botulinum	III	48212	CD	SRS1009714	(37, 38)
C. botulinum	III	43243	CD	SRS1009712	(37, 38)
C. botulinum	III	38028	CD	SRS1009711	(37, 38)
C. botulinum	III	29401	CD	SRR2124863	(37, 38)
C. botulinum	III	12LNR13	CD	SRR2124859	(37, 38)
C. botulinum	III	12LNR10	CD	SRR2124854	(37, 38)
C. botulinum	III	12LNRI	CD	SRR2120181	(37, 38)
C. botulinum	III	Sp77	CD	NZ_JENQ01000145.1	(49)
C. botulinum	III	BKT2873	CD	NZ_CM003340.1	(49)
C. botulinum	III	BKT75002	CD	NZ_CM003339.1	(49)
C. botulinum	III	V891	CD	NZ_CM003321.1	(49)
C. botulinum	III	BKT015925	CD	NC_015417.1	(8)
C. botulinum	III	EklundC	CD	ABDQ00000000	(49, 75)
C. botulinum	III	003-9	CD		(38)
C. botulinum	III	6813	CD		(38)
C. botulinum	III	80671III	CD		(38)
C. botulinum	III	4622	CD		(38)
C. botulinum	III	31354	CD		(38)
C. botulinum	III	32150	CD		(38)
C. botulinum	III	32670	CD		(38)
C. botulinum	III	2286-3	CD		(38)
C. botulinum	III	4456-11	CD		(38)
C. botulinum	III	9-2 322	CD		(38)
C. botulinum	III	RKI-6	CD		(38)
C. botulinum	III	07-V891	CD		(38)
C. botulinum	III	621125	CD		(38)
C. botulinum	III	08BKT15925	CD		(38)
C. botulinum	III	13LNR1	CD		(38)
C. botulinum	III	6136-A-12	CD		(38)
C. botulinum	III	30607	CD		(38)
C. botulinum	III	48751	CD		(38)
C. botulinum	III	52859	CD		(38)
C. botulinum	III	60979	CD		(38)

C. botulinum	III	65304	CD		(38)
C. botulinum	III	NRCB1	CD		(38)
C. botulinum	III	NRCB2	CD		(38)
C. botulinum	III	NRCB3	CD		(38)
C. botulinum	III	92962XIV	CD		(38)
C. botulinum	III	481290	CD		(38)
C. botulinum	III	13451II	CD		(38)
C. botulinum	III	13451XII	CD		(38)
C. botulinum	III	81290	CD		(38)
C. botulinum	III	24992-1	CD		(38)
C. botulinum	III	97371	CD		(38)
C. botulinum	III	37393	CD		(38)
C. botulinum	III	38997	CD		(38)
C. botulinum	III	12792	CD		(38)
C. botulinum	III	5391-09	CD		(38)
C. botulinum	III	5674-10	CD		(38)
C. botulinum	III	12LNR8	CD		(38)
C. botulinum	III	ISS_Animal-2A	CD		(38)
C. botulinum	III	ISS_Animal-2B	CD		(38)
C. botulinum	III	276	CD		(38)
C. botulinum	III	72870	CD		(38)
C. botulinum	III	RKI-3	CD		(38)
C. botulinum	III	RKI-4	CD		(38)
C. botulinum	III	RKI-5	CD		(38)
C. botulinum	III	16868	D	NZ_CM003334.1	(49)
C. botulinum	III	1873	D	ACSJ00000000.1	(49)
C. botulinum	III	RKI-7	D		(38)
C. botulinum	III	2639-2	D		(38)
C. botulinum	III	16878	D		(38)
C. botulinum	III	16983	D		(38)
C. botulinum	III	51714	DC	SRS1009737	(37, 38)
C. botulinum	III	47295	DC	SRS1009736	(37, 38)
C. botulinum	III	LNC5	DC	SRS1009735	(37, 38)
C. botulinum	III	CP05	DC	MVJC00000000	(76)
C. botulinum	III	1277	DC	MVJB00000000	(76)
C. botulinum	III	1276	DC	MVJA00000000	(76)
C. botulinum	III	1275	DC	MVIZ00000000	(76)
C. botulinum	III	1274	DC	MVIY00000000	(76)
C. botulinum	III	DC5	DC	JDRY00000000.1	(49)
C. botulinum	III	4456-11	DC		(38)
C. botulinum	III	Gun	DC		(38)
C. botulinum	III	7296	DC		(38)
C. botulinum	III	7357	DC		(38)
C. botulinum	III	OFD05	DC		(38)
C. botulinum	III	16564	DC		(38)
C. botulinum	III	NCTC8265	DC		(38)
C. botulinum	III	564424	DC		(38)
C. botulinum	III	96564	DC		(38)

C. botulinum	III	86469	DC		(38)
C. botulinum	III	18128	DC		(38)
C. botulinum	III	1585-18-11	DC		(38)
C. botulinum	III	SP28	DC		(38)
C. botulinum	III	1585-19-11	DC		(38)
C. botulinum	III	360	DC		(38)
C. botulinum	III	LNC2	DC		(38)
C. botulinum	III	RKI-8	DC		(38)
C. botulinum	III	RKI-9	DC		(38)
C. botulinum	III	RKI-10	DC		(38)
C. botulinum	IV	117	G		(9, 10)
C. botulinum	IV	2739	G		(9, 10)
C. botulinum	IV	2740	G		(9, 10)
C. botulinum	IV	2741	G		(9, 10)
C. botulinum	IV	2742	G		(9, 10)
C. botulinum	IV	89G	G	CP014175	(15)
C. sporogenes		Prevot 1662	B	LAGM01000000	(54)
C. sporogenes		ATCC 51387	B	LAGD01000000	(54)
C. butyricum		CDC51208	E	CP13238	(45)
C. butyricum		ATCC 43181	E		(43)
C. butyricum		ATCC 43755	E		(43)
C. baratii		CDC51267	F	CP014203	(45)

Works Cited

1. Carroll AC, Wong A. Plasmid persistence: costs, benefits, and the plasmid paradox. Can J Microbiol. 2018;64(5):293-304. Epub 2018/03/22. doi: 10.1139/cjm-2017-0609. PubMed PMID: 29562144.
2. Harrison E, Brockhurst MA. Plasmid-mediated horizontal gene transfer is a coevolutionary process. Trends Microbiol. 2012;20(6):262-7. Epub 2012/05/09. doi: 10.1016/j.tim.2012.04.003. PubMed PMID: 22564249.
3. Eberhard WG. Why do bacterial plasmids carry some genes and not others? Plasmid. 1989;21(3):167-74. Epub 1989/05/01. PubMed PMID: 2675150.
4. Rudkin JK, McLoughlin RM, Preston A, Massey RC. Bacterial toxins: Offensive, defensive, or something else altogether? PLoS pathogens. 2017;13(9):e1006452. Epub 2017/09/22. doi: 10.1371/journal.ppat.1006452. PubMed PMID: 28934339; PMCID: PMC5608399.
5. Pilla G, Tang CM. Going around in circles: virulence plasmids in enteric pathogens. Nat Rev Microbiol. 2018;16(8):484-95. Epub 2018/06/02. doi: 10.1038/s41579-018-0031-2. PubMed PMID: 29855597.
6. Bruggemann H. Genomics of clostridial pathogens: implication of extrachromosomal elements in pathogenicity. Curr Opin Microbiol. 2005;8(5):601-5. Epub 2005/08/30. doi: 10.1016/j.mib.2005.08.006. PubMed PMID: 16125440.
7. Peck MW, Stringer SC, Carter AT. *Clostridium botulinum* in the post-genomic era. Food Microbiol. 2011;28(2):183-91. Epub 2011/02/15. doi: 10.1016/j.fm.2010.03.005. PubMed PMID: 21315972.
8. Skarin H, Hafstrom T, Westerberg J, Segerman B. *Clostridium botulinum* group III: a group with dual identity shaped by plasmids, phages and mobile elements. BMC Genomics. 2011;12:185. Epub 2011/04/14. doi: 10.1186/1471-2164-12-185. PubMed PMID: 21486474; PMCID: PMC3098183.
9. Zhou Y, Sugiyama H, Nakano H, Johnson EA. The genes for the *Clostridium botulinum* type G toxin complex are on a plasmid. Infect Immun. 1995;63(5):2087-91. Epub 1995/05/01. PubMed PMID: 7729925; PMCID: PMC173270.
10. Eklund MW, Poysky FT, Mseitif LM, Strom MS. Evidence for plasmid-mediated toxin and bacteriocin production in *Clostridium botulinum* type G. Appl Environ Microbiol. 1988;54(6):1405-8. Epub 1988/06/01. PubMed PMID: 2843093; PMCID: PMC202669.
11. Weickert MJ, Chambliss GH, Sugiyama H. Production of toxin by *Clostridium botulinum* type A strains cured by plasmids. Appl Environ Microbiol. 1986;51(1):52-6. Epub 1986/01/01. PubMed PMID: 3082278; PMCID: PMC238814.
12. Marshall KM, Bradshaw M, Pellett S, Johnson EA. Plasmid encoded neurotoxin genes in *Clostridium botulinum* serotype A subtypes. Biochem Biophys Res Commun. 2007;361(1):49-54. Epub 2007/07/31. doi: 10.1016/j.bbrc.2007.06.166. PubMed PMID: 17658467; PMCID: PMC2346372.
13. Zhang Z, Hintsa H, Chen Y, Korkeala H, Lindstrom M. Plasmid-borne type E neurotoxin gene clusters in *Clostridium botulinum* strains. Appl Environ Microbiol. 2013;79(12):3856-9. Epub 2013/04/09. doi: 10.1128/AEM.00080-13. PubMed PMID: 23563942; PMCID: PMC3675925.
14. Carter AT, Austin JW, Weedmark KA, Corbett C, Peck MW. Three classes of plasmid (47-63 kb) carry the type B neurotoxin gene cluster of group II *Clostridium botulinum*. Genome Biol Evol. 2014;6(8):2076-87. Epub 2014/08/01. doi: 10.1093/gbe/evu164. PubMed PMID: 25079343; PMCID: PMC4231633.

15. Halpin JL, Hill K, Johnson SL, Bruce DC, Shirey TB, Dykes JK, Luquez C. Finished Whole-Genome Sequence of C*lostridium argentinense* Producing Botulinum Neurotoxin Type G. Genome Announc. 2017;5(21). Epub 2017/05/27. doi: 10.1128/genomeA.00380-17. PubMed PMID: 28546482; PMCID: PMC5477395.
16. Smith TJ, Hill KK, Xie G, Foley BT, Williamson CHD, Foster JT, Johnson SL, Chertkov O, Teshima H, Gibbons HS, Johnsky LA, Karavis MA, Smith LA. Genomic sequences of six botulinum neurotoxin-producing strains representing three clostridial species illustrate the mobility and diversity of botulinum neurotoxin genes. Infect Genet Evol. 2015;30:102-13. Epub 2014/12/10. doi: 10.1016/j.meegid.2014.12.002. PubMed PMID: 25489752; PMCID: PMC5459376.
17. Tatusov RL, Galperin MY, Natale DA, Koonin EV. The COG database: a tool for genome-scale analysis of protein functions and evolution. Nucleic Acids Res. 2000;28(1):33-6. Epub 1999/12/11. PubMed PMID: 10592175; PMCID: PMC102395.
18. Ainsworth S, Stockdale S, Bottacini F, Mahony J, van Sinderen D. The *Lactococcus lactis* plasmidome: much learnt, yet still lots to discover. FEMS Microbiol Rev. 2014;38(5):1066-88. Epub 2014/05/28. doi: 10.1111/1574-6976.12074. PubMed PMID: 24861818.
19. de Toro M, Garcillaon-Barcia MP, De La Cruz F. Plasmid Diversity and Adaptation Analyzed by Massive Sequencing of *Escherichia coli* Plasmids. Microbiol Spectr. 2014;2(6). Epub 2015/06/25. doi: 10.1128/microbiolspec.PLAS-0031-2014. PubMed PMID: 26104438.
20. San Millan A, MacLean RC. Fitness Costs of Plasmids: a Limit to Plasmid Transmission. Microbiol Spectr. 2017;5(5). Epub 2017/09/26. doi: 10.1128/microbiolspec.MTBP-0016-2017. PubMed PMID: 28944751.
21. Harrison PW, Lower RP, Kim NK, Young JP. Introducing the bacterial 'chromid': not a chromosome, not a plasmid. Trends Microbiol. 2010;18(4):141-8. Epub 2010/01/19. doi: 10.1016/j.tim.2009.12.010. PubMed PMID: 20080407.
22. diCenzo GC, Finan TM. The Divided Bacterial Genome: Structure, Function, and Evolution. Microbiol Mol Biol Rev. 2017;81(3). Epub 2017/08/11. doi: 10.1128/MMBR.00019-17. PubMed PMID: 28794225; PMCID: PMC5584315.
23. Scalfaro C, Iacobino A, Grande L, Morabito S, Franciosa G. Effects of Megaplasmid Loss on Growth of Neurotoxigenic *Clostridium butyricum* Strains and Botulinum Neurotoxin Type E Expression. Front Microbiol. 2016;7:217. Epub 2016/03/05. doi: 10.3389/fmicb.2016.00217. PubMed PMID: 26941734; PMCID: PMC4766289.
24. Wu ST, Zhu ZW, Fu LM, Niu BF, Li WZ. WebMGA: a customizable web server for fast metagenomic sequence analysis. BMC Genomics. 2011;12:444. Epub 2011/09/09. doi: Artn 444 10.1186/1471-2164-12-444. PubMed PMID: WOS:000295280500002; PMCID: PMC3180703.
25. Smith TJ, Hill KK, Foley BT, Detter JC, Munk AC, Bruce DC, Doggett NA, Smith LA, Marks JD, Xie G, Brettin TS. Analysis of the neurotoxin complex genes in *Clostridium botulinum* A1-A4 and B1 strains: BoNT/A3, /Ba4 and /B1 clusters are located within plasmids. PLoS One. 2007;2(12):e1271. Epub 2007/12/07. doi: 10.1371/journal.pone.0001271. PubMed PMID: 18060065; PMCID: PMC2092393.
26. Franciosa G, Maugliani A, Scalfaro C, Aureli P. Evidence that plasmid-borne botulinum neurotoxin type B genes are widespread among *Clostridium botulinum* serotype B strains. PLoS One. 2009;4(3):e4829. Epub 2009/03/17. doi: 10.1371/journal.pone.0004829. PubMed PMID: 19287483; PMCID: PMC2653641.
27. Dover N, Barash JR, Hill KK, Davenport KW, Teshima H, Xie G, Arnon SS. *Clostridium botulinum* strain Af84 contains three neurotoxin gene clusters: *bont/A2*, *bont/F4* and *bont/F5*.

PLoS One. 2013;8(4):e61205. Epub 2013/05/03. doi: 10.1371/journal.pone.0061205. PubMed PMID: 23637798; PMCID: PMC3625220.
28. Raphael BH, Bradshaw M, Kalb SR, Joseph LA, Luquez C, Barr JR, Johnson EA, Maslanka SE. *Clostridium botulinum* strains producing BoNT/F4 or BoNT/F5. Appl Environ Microbiol. 2014;80(10):3250-7. Epub 2014/03/19. doi: 10.1128/AEM.00284-14. PubMed PMID: 24632257; PMCID: PMC4018930.
29. Umeda K, Seto Y, Kohda T, Mukamoto M, Kozaki S. Stability of toxigenicity in proteolytic *Clostridium botulinum* type B upon serial passage. Microbiology and immunology. 2012;56(5):338-41. Epub 2012/02/23. doi: 10.1111/j.1348-0421.2012.00441.x. PubMed PMID: 22352877.
30. Hosomi K, Sakaguchi Y, Kohda T, Gotoh K, Motooka D, Nakamura S, Umeda K, Iida T, Kozaki S, Mukamoto M. Complete nucleotide sequence of a plasmid containing the botulinum neurotoxin gene in *Clostridium botulinum* type B strain 111 isolated from an infant patient in Japan. Mol Genet Genomics. 2014;289(6):1267-74. Epub 2014/08/26. doi: 10.1007/s00438-014-0887-4. PubMed PMID: 25149145.
31. Umeda K, Seto Y, Kohda T, Mukamoto M, Kozaki S. Genetic characterization of *Clostridium botulinum* associated with type B infant botulism in Japan. J Clin Microbiol. 2009;47(9):2720-8. Epub 2009/07/03. doi: 10.1128/JCM.00077-09. PubMed PMID: 19571018; PMCID: PMC2738102.
32. McCallum N, Gray TJ, Wang Q, Ng J, Hicks L, Nguyen T, Yuen M, Hill-Cawthorne GA, Sintchenko V. Genomic Epidemiology of *Clostridium botulinum* Isolates from Temporally Related Cases of Infant Botulism in New South Wales, Australia. J Clin Microbiol. 2015;53(9):2846-53. Epub 2015/06/26. doi: 10.1128/JCM.00143-15. PubMed PMID: 26109442; PMCID: PMC4540925.
33. Fillo S, Giordani F, Anselmo A, Fortunato A, Palozzi AM, De Santis R, Ciammaruconi A, Spagnolo F, Anniballi F, Fiore A, Auricchio B, De Medici D, Lista F. Draft Genome Sequence of *Clostridium botulinum* B2 450 Strain from Wound Botulism in a Drug User in Italy. Genome Announc. 2015;3(2). Epub 2015/04/04. doi: 10.1128/genomeA.00238-15. PubMed PMID: 25838491; PMCID: PMC4384495.
34. Strom MS, Eklund MW, Poysky FT. Plasmids in *Clostridium botulinum* and related *Clostridium* species. Appl Environ Microbiol. 1984;48(5):956-63. Epub 1984/11/01. PubMed PMID: 6391384; PMCID: PMC241657.
35. Hill KK, Xie G, Foley BT, Smith TJ, Munk AC, Bruce D, Smith LA, Brettin TS, Detter JC. Recombination and insertion events involving the botulinum neurotoxin complex genes in *Clostridium botulinum* types A, B, E and F and *Clostridium butyricum* type E strains. BMC Biol. 2009;7:66. Epub 2009/10/07. doi: 10.1186/1741-7007-7-66. PubMed PMID: 19804621; PMCID: PMC2764570.
36. Carter AT, Austin JW, Weedmark KA, Peck MW. Evolution of Chromosomal *Clostridium botulinum* Type E Neurotoxin Gene Clusters: Evidence Provided by Their Rare Plasmid-Borne Counterparts. Genome Biol Evol. 2016;8(3):540-55. Epub 2016/03/05. doi: 10.1093/gbe/evw017. PubMed PMID: 26936890; PMCID: PMC4824171.
37. Woudstra C, Le Marechal C, Souillard R, Bayon-Auboyer MH, Mermoud I, Desoutter D, Fach P. New Insights into the Genetic Diversity of *Clostridium botulinum* Group III through Extensive Genome Exploration. Front Microbiol. 2016;7:757. Epub 2016/06/01. doi: 10.3389/fmicb.2016.00757. PubMed PMID: 27242769; PMCID: PMC4871853.
38. Woudstra C, Le Marechal C, Souillard R, Anniballi F, Auricchio B, Bano L, Bayon-Auboyer MH, Koene M, Mermoud I, Brito RB, Lobato FCF, Silva ROS, Dorner MB, Fach P. Investigation of *Clostridium botulinum* group III's mobilome content. Anaerobe. 2018;49:71-7.

Epub 2017/12/31. doi: 10.1016/j.anaerobe.2017.12.009. PubMed PMID: WOS:000428487900012.
39. Campbell K, Collins MD, East AK. Nucleotide sequence of the gene coding for *Clostridium botulinum* (*Clostridium argentinense*) type G neurotoxin: genealogical comparison with other clostridial neurotoxins. Biochim Biophys Acta. 1993;1216(3):487-91. Epub 1993/12/14. PubMed PMID: 8268233.
40. Sonnabend O, Sonnabend W, Heinzle R, Sigrist T, Dirnhofer R, Krech U. Isolation of *Clostridium botulinum* type G and identification of type G botulinal toxin in humans: report of five sudden unexpected deaths. J Infect Dis. 1981;143(1):22-7. Epub 1981/01/01. PubMed PMID: 7012244.
41. Ciccarelli AS, Whaley DN, McCroskey LM, Gimenez DF, Dowell VR, Jr., Hatheway CL. Cultural and physiological characteristics of *Clostridium botulinum* type G and the susceptibility of certain animals to its toxin. Appl Environ Microbiol. 1977;34(6):843-8. Epub 1977/12/01. PubMed PMID: 74236; PMCID: PMC242758.
42. Sonnabend WF, Sonnabend UP, Krech T. Isolation of *Clostridium botulinum* type G from Swiss soil specimens by using sequential steps in an identification scheme. Appl Environ Microbiol. 1987;53(8):1880-4. Epub 1987/08/01. PubMed PMID: 3116935; PMCID: PMC204018.
43. Hauser D, Gibert M, Boquet P, Popoff MR. Plasmid localization of a type E botulinal neurotoxin gene homologue in toxigenic *Clostridium butyricum* strains, and absence of this gene in non-toxigenic *C. butyricum* strains. FEMS Microbiol Lett. 1992;78(2-3):251-5. Epub 1992/12/01. PubMed PMID: 1490606.
44. Iacobino A, Scalfaro C, Franciosa G. Structure and genetic content of the megaplasmids of neurotoxigenic *Clostridium butyricum* type E strains from Italy. PLoS One. 2013;8(8):e71324. Epub 2013/08/24. doi: 10.1371/journal.pone.0071324. PubMed PMID: 23967192; PMCID: PMC3744543.
45. Halpin JL, Hill K, Johnson SL, Bruce DC, Shirey TB, Dykes JK, Luquez C. Finished Whole-Genome Sequences of *Clostridium butyricum* Toxin Subtype E4 and *Clostridium baratii* Toxin Subtype F7 Strains. Genome Announc. 2017;5(29). Epub 2017/07/22. doi: 10.1128/genomeA.00375-17. PubMed PMID: 28729254; PMCID: PMC5522921.
46. Gimenez JA, Gimenez MA, DasGupta BR. Characterization of the neurotoxin isolated from a *Clostridium baratii* strain implicated in infant botulism. Infect Immun. 1992;60(2):518-22. Epub 1992/02/01. PubMed PMID: 1730484; PMCID: PMC257658.
47. Marshall KM, Bradshaw M, Johnson EA. Conjugative botulinum neurotoxin-encoding plasmids in *Clostridium botulinum*. PLoS One. 2010;5(6):e11087. Epub 2010/06/17. doi: 10.1371/journal.pone.0011087. PubMed PMID: 20552020; PMCID: PMC2884020.
48. Nawrocki EM, Bradshaw M, Johnson EA. Botulinum neurotoxin-encoding plasmids can be conjugatively transferred to diverse clostridial strains. Scientific reports. 2018;8(1):3100. Epub 2018/02/17. doi: 10.1038/s41598-018-21342-9. PubMed PMID: 29449580; PMCID: PMC5814558.
49. Skarin H, Segerman B. Plasmidome interchange between *Clostridium botulinum*, *Clostridium novyi* and *Clostridium haemolyticum* converts strains of independent lineages into distinctly different pathogens. PLoS One. 2014;9(9):e107777. Epub 2014/09/26. doi: 10.1371/journal.pone.0107777. PubMed PMID: 25254374; PMCID: PMC4177856.
50. Fujii N, Kimura K, Yokosawa N, Oguma K, Yashiki T, Takeshi K, Ohyama T, Isogai E, Isogai H. Similarity in nucleotide sequence of the gene encoding nontoxic component of botulinum toxin produced by toxigenic *Clostridium butyricum* strain BL6340 and *Clostridium*

botulinum type E strain Mashike. Microbiology and immunology. 1993;37(5):395-8. Epub 1993/01/01. PubMed PMID: 8355622.
51. Zhou Y, Sugiyama H, Johnson EA. Transfer of neurotoxigenicity from *Clostridium butyricum* to a nontoxigenic *Clostridium botulinum* type E-like strain. Appl Environ Microbiol. 1993;59(11):3825-31. Epub 1993/11/01. PubMed PMID: 8285687; PMCID: PMC182537.
52. Dover N, Barash JR, Hill KK, Xie G, Arnon SS. Molecular characterization of a novel botulinum neurotoxin type H gene. J Infect Dis. 2014;209(2):192-202. Epub 2013/10/10. doi: 10.1093/infdis/jit450. PubMed PMID: 24106295.
53. Williamson CHD, Sahl JW, Smith TJ, Xie G, Foley BT, Smith LA, Fernandez RA, Lindstrom M, Korkeala H, Keim P, Foster J, Hill K. Comparative genomic analyses reveal broad diversity in botulinum-toxin-producing Clostridia. Bmc Genomics. 2016;17(1):180. Epub 2016/03/05. doi: ARTN 180
10.1186/s12864-016-2502-z. PubMed PMID: WOS:000371595000006; PMCID: PMC4778365.
54. Weigand MR, Pena-Gonzalez A, Shirey TB, Broeker RG, Ishaq MK, Konstantinidis KT, Raphael BH. Implications of Genome-Based Discrimination between *Clostridium botulinum* Group I and *Clostridium sporogenes* Strains for Bacterial Taxonomy. Appl Environ Microbiol. 2015;81(16):5420-9. Epub 2015/06/07. doi: 10.1128/AEM.01159-15. PubMed PMID: 26048939; PMCID: PMC4510194.
55. Carter AT, Stringer SC, Webb MD, Peck MW. The type F6 neurotoxin gene cluster locus of group II *Clostridium botulinum* has evolved by successive disruption of two different ancestral precursors. Genome Biol Evol. 2013;5(5):1032-7. Epub 2013/05/07. doi: 10.1093/gbe/evt068. PubMed PMID: 23645598; PMCID: PMC3673618.
56. Carter AT, Peck MW. Genomes, neurotoxins and biology of *Clostridium botulinum* Group I and Group II. Res Microbiol. 2015;166(4):303-17. Epub 2014/12/03. doi: 10.1016/j.resmic.2014.10.010. PubMed PMID: 25445012; PMCID: PMC4430135.
57. Zhang S, Lebreton F, Mansfield MJ, Miyashita SI, Zhang J, Schwartzman JA, Tao L, Masuyer G, Martinez-Carranza M, Stenmark P, Gilmore MS, Doxey AC, Dong M. Identification of a Botulinum Neurotoxin-like Toxin in a Commensal Strain of *Enterococcus faecium*. Cell host & microbe. 2018;23(2):169-76 e6. Epub 2018/02/06. doi: 10.1016/j.chom.2017.12.018. PubMed PMID: 29396040; PMCID: PMC5926203.
58. Freitas AR, Tedim AP, Francia MV, Jensen LB, Novais C, Peixe L, Sanchez-Valenzuela A, Sundsfjord A, Hegstad K, Werner G, Sadowy E, Hammerum AM, Garcia-Migura L, Willems RJ, Baquero F, Coque TM. Multilevel population genetic analysis of *vanA* and *vanB* *Enterococcus faecium* causing nosocomial outbreaks in 27 countries (1986-2012). J Antimicrob Chemother. 2016;71(12):3351-66. Epub 2016/08/18. doi: 10.1093/jac/dkw312. PubMed PMID: 27530756.
59. Van Tyne D, Gilmore MS. Virulence Plasmids of Nonsporulating Gram-Positive Pathogens. Microbiology Spectrum. 2014;2(5). Epub 2014/12/30. doi: 10.1128/microbiolspec.PLAS-0002-2013. PubMed PMID: 25544937; PMCID: PMC4275793.
60. Rossetto O, Pirazzini M, Montecucco C. Botulinum neurotoxins: genetic, structural and mechanistic insights. Nat Rev Microbiol. 2014;12(8):535-49. Epub 2014/07/01. doi: 10.1038/nrmicro3295. PubMed PMID: 24975322.
61. Montecucco C, Rasotto MB. On botulinum neurotoxin variability. MBio. 2015;6(1). Epub 2015/01/08. doi: 10.1128/mBio.02131-14. PubMed PMID: 25564463; PMCID: PMC4313909.
62. Hulter N, Ilhan J, Wein T, Kadibalban AS, Hammerschmidt K, Dagan T. An evolutionary perspective on plasmid lifestyle modes. Curr Opin Microbiol. 2017;38:74-80. Epub 2017/05/26. doi: 10.1016/j.mib.2017.05.001. PubMed PMID: 28538166.

63. Jackson RW, Vinatzer B, Arnold DL, Dorus S, Murillo J. The influence of the accessory genome on bacterial pathogen evolution. Mob Genet Elements. 2011;1(1):55-65. Epub 2011/10/22. doi: 10.4161/mge.1.1.16432. PubMed PMID: 22016845; PMCID: PMC3190274.
64. Benoit RM. Botulinum Neurotoxin Diversity from a Gene-Centered View. Toxins. 2018;10(8). Epub 2018/08/04. doi: 10.3390/toxins10080310. PubMed PMID: 30071587; PMCID: PMC6115791.
65. Shukla HD, Sharma SK. *Clostridium botulinum*: a bug with beauty and weapon. Critical reviews in microbiology. 2005;31(1):11-8. Epub 2005/04/21. doi: 10.1080/10408410590912952. PubMed PMID: 15839401.
66. Adams V, Li J, Wisniewski JA, Uzal FA, Moore RJ, McClane BA, Rood JI. Virulence Plasmids of Spore-Forming Bacteria. Microbiol Spectr. 2014;2(6). Epub 2015/06/25. doi: 10.1128/microbiolspec.PLAS-0024-2014. PubMed PMID: 26104459.
67. Halpin AL, Khouri JM, Payne JR, Nakao JH, Cronquist A, Kalas N, Mohr M, Osborne M, O'Dell S, Luquez C, Klontz KC, Sobel J, Rao AK. Type F Infant Botulism: Investigation of Recent Clusters and Overview of This Exceedingly Rare Disease. Clin Infect Dis. 2017;66(suppl_1):S92-S4. Epub 2018/01/03. doi: 10.1093/cid/cix818. PubMed PMID: 29293930.
68. Peck MW, Smith TJ, Anniballi F, Austin JW, Bano L, Bradshaw M, Cuervo P, Cheng LW, Derman Y, Dorner BG, Fisher A, Hill KK, Kalb SR, Korkeala H, Lindstrom M, Lista F, Luquez C, Mazuet C, Pirazzini M, Popoff MR, Rossetto O, Rummel A, Sesardic D, Singh BR, Stringer SC. Historical Perspectives and Guidelines for Botulinum Neurotoxin Subtype Nomenclature. Toxins. 2017;9(1). Epub 2017/01/21. doi: 10.3390/toxins9010038. PubMed PMID: 28106761; PMCID: PMC5308270.
69. Negahdaripour M, Nezafat N, Hajighahramani N, Rahmatabadi SS, Ghasemi Y. Investigating CRISPR-Cas systems in *Clostridium botulinum* via bioinformatics tools. Infect Genet Evol. 2017;54:355-73. Epub 2017/07/08. doi: 10.1016/j.meegid.2017.06.027. PubMed PMID: 28684374.
70. Kenri T, Sekizuka T, Yamamoto A, Iwaki M, Komiya T, Hatakeyama T, Nakajima H, Takahashi M, Kuroda M, Shibayama K. Genetic characterization and comparison of *Clostridium botulinum* isolates from botulism cases in Japan between 2006 and 2011. Appl Environ Microbiol. 2014;80(22):6954-64. Epub 2014/09/07. doi: 10.1128/AEM.02134-14. PubMed PMID: 25192986; PMCID: PMC4249013.
71. Giordani F, Fillo S, Anselmo A, Palozzi AM, Fortunato A, Gentile B, Azarnia Tehran D, Ciammaruconi A, Spagnolo F, Pittiglio V, Anniballi F, Auricchio B, De Medici D, Lista F. Genomic characterization of Italian *Clostridium botulinum* group I strains. Infect Genet Evol. 2015;36:62-71. Epub 2015/09/06. doi: 10.1016/j.meegid.2015.08.042. PubMed PMID: 26341861.
72. Sakaguchi Y, Hosomi K, Uchiyama J, Ogura Y, Umeda K, Sakaguchi M, Kohda T, Mukamoto M, Misawa N, Matsuzaki S, Hayashi T, Kozaki S. Draft Genome Sequence of *Clostridium botulinum* Type B Strain Osaka05, Isolated from an Infant Patient with Botulism in Japan. Genome Announc. 2014;2(1). Epub 2014/01/25. doi: 10.1128/genomeA.01010-13. PubMed PMID: 24459252; PMCID: PMC3900884.
73. Williamson CHD, Vazquez AJ, Hill K, Smith TJ, Nottingham R, Stone NE, Sobek CJ, Cocking JH, Fernandez RA, Caballero PA, Leiser OP, Keim P, Sahl JW. Differentiating Botulinum Neurotoxin-Producing Clostridia with a Simple, Multiplex PCR Assay. Applied and Environmental Microbiology. 2017;83(18). Epub 2017/07/25. doi: UNSP e00806 10.1128/AEM.00806-17. PubMed PMID: WOS:000408730900004.

74. Stringer SC, Carter AT, Webb MD, Wachnicka E, Crossman LC, Sebaihia M, Peck MW. Genomic and physiological variability within Group II (non-proteolytic) *Clostridium botulinum*. BMC Genomics. 2013;14:333. Epub 2013/05/18. doi: 10.1186/1471-2164-14-333. PubMed PMID: 23679073; PMCID: PMC3672017.
75. Hassan KA, Tetu SG, Elbourne LD, Johnson EA, Paulsen IT. Genome Sequence of the Group III *Clostridium botulinum* Strain Eklund-C. Genome Announc. 2013;1(2):e0004413. Epub 2013/03/22. doi: 10.1128/genomeA.00044-13. PubMed PMID: 23516187; PMCID: PMC3622972.
76. Woudstra C, Brito RB, Fonseca Júnior AA, Silva ROS, Lobato FCF, Fach P. Draft Genome Sequences of Five Brazilian *Clostridium botulinum* Group III Type D/C Strains. Genome Announcements. 2017;5(20). Epub 2017/05/20. doi: 10.1128/genomeA.00349-17. PubMed PMID: 28522711; PMCID: PMC5442381.

Chapter 1: Exploring the host range of a BoNT-encoding plasmid

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Author Contributions

E.M.N. designed and conducted experiments and drafted the manuscript with input from all authors. M.B. generated the initial Em^R plasmid donor strain and performed PFGE analysis and Southern hybridization. E.A.J. assisted in the supervision and critique of the research.

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Additional Information

The authors declare that they have no conflicts of interest.

Abstract

Most Group I *Clostridium botulinum* strains harbor botulinum neurotoxin (*bont*) genes on their chromosome, while some carry these genes (including *bont/a*, *bont/b*, and *bont/f*) on large plasmids. Prior work in our laboratory demonstrated that Group I BoNT plasmids were mobilized to *C. botulinum* recipient strains containing the Tn*916* transposon. Here, we show that Tn*916* is nonessential for plasmid transfer. Relying on an auxotrophic donor phenotype and a plasmid-borne selectable marker, we observed the transfer of pCLJ, a 270 kb plasmid harboring two *bont* genes, from its host strain to various clostridia. Transfer frequency was greatest to other Group I *C. botulinum* strains, but the plasmid was also transferred into traditionally nontoxigenic species, namely *C. sporogenes* and *C. butyricum*. Expression and toxicity of BoNT/A4 was confirmed in transconjugants by immunoblot and mouse bioassay. These data indicate that conjugation within the genus *Clostridium* can occur across physiological Groups of *C. botulinum*, supporting horizontal gene transfer via *bont*-bearing plasmids. The transfer of plasmids possessing *bont* genes to resistant *Clostridium* spp. such as *C. sporogenes* could impact biological safety for animals and humans. These plasmids may play an environmental role in initiating death in vertebrates, leading to decomposition and nutrient recycling of animal biomass.

Introduction

Clostridium is a diverse genus whose members are gram-positive, spore-forming, anaerobic bacteria. The species *C. botulinum* is notable for its production of botulinum neurotoxins (BoNTs), zinc-dependent proteases that specifically cleave SNARE proteins, preventing the release of neurotransmitters from synaptic vesicles (1). BoNTs attack "the most vulnerable synapses" (2) including the hemidiaphragm, leading to respiratory arrest and death. BoNTs are categorized into seven serotypes, designated A through G on the basis of their neutralization against death in mice with homologous antisera. The diverse *Clostridium* strains expressing the various BoNT serotypes are classified into four physiological Groups, designated with numerals I-IV. Group I encompasses proteolytic strains producing BoNT/A, /B, and /F, which are responsible for the vast majority of human botulism cases in the USA (3). Historically, BoNT formation and lethality testing in mice was the sole criterion required for designation of the species *C. botulinum*, a framework that has resulted in the complicated genome-based phylogeny of the species today (4). The availability of sequence information has revealed the extensive genetic distance between the four Groups.

The genomic sequences from BoNT-producing clostridia have also helped to discern important phenotypic properties. These include environmental, nutritional, and molecular mechanisms governing BoNT expression, proteolytic processing and activation, cell lysis and release of BoNT, and stability of BoNT in culture (5, 6). Genomic studies have also been important in revealing and understanding atypical neurotoxigenic strains, such as those that possess two (or three) BoNT gene clusters, express more than one BoNT in culture, produce chimeric BoNTs including BoNT/FA, or encode BoNT-like molecules in non-clostridial organisms (7-9). Sequence comparisons indicate that *C. botulinum* strains and BoNTs have

evolved independently of one another, suggesting that horizontal gene transfer may play a role in toxin acquisition (10-12).

Further evidence for horizontal gene transfer in *C. botulinum* is given by the abundance of genes for mobile elements surrounding the BoNT gene clusters (13). All *bont* genes are encoded as part of a conserved cluster, where they are found linked to genes encoding accessory or non-toxic complex proteins. In most sequenced strains, *bont* gene clusters are located in specific positions on the chromosome or plasmid. Analyses of the neighboring regions often identify nearby elements that may be involved in recombination or other functions, including flagellin genes and IS elements (14). Intact or partial flagellin genes are present upstream of the cluster in some strains, and are frequently hypervariable sites of recombination in other species (15-18). Both plasmid-borne and chromosomally located toxin gene clusters are flanked by mostly defective IS elements of various families. When intact, IS elements encode transposase enzymes that promote their own translocation. The IS elements identified near the toxin gene clusters in Group I strains have so far been shown to be genetically degraded, sharing a maximum of 83% amino acid homology with the full-length elements (19). The recombination events that inserted toxin clusters into their modern loci are therefore assumed to have occurred in the distant past.

Weickert *et al.* detected plasmids in *C. botulinum* type A strains; however, strains cured of plasmids still produced BoNT (20). The first report of plasmid-borne BoNT genes in Group I *C. botulinum* strains was made in 2007 (21), and was confirmed by Smith *et al.* in genomic sequencing studies (19). Despite recent advances in clostridial plasmid biology, particularly in *Clostridium perfringens* (22), the BoNT plasmids are poorly characterized. In Group I *C. botulinum* strains, they range in size from approximately 140-270 kb and encode BoNTs of serotypes A, B, F, or multiple BoNTs (19, 23, 24). Although comparatively few completed

sequences are available, the presence of *bont* genes on plasmids is likely widespread; one survey found *bont/b* on a plasmid in 32 of 60 strains examined, including 21 of 22 *bont/b1* strains (23). A recent study extends this observation, showing similar plasmids harboring *bont*/*b6* present in strains of both the *C. botulinum* and *C. sporogenes* chromosomal lineages (12). Multi-domain homologues of BoNTs were also newly identified in a separate class of Firmicutes, raising questions about the origin and distribution of the neurotoxin (7). Expression of one such homolog, BoNT/Wo, reveals that the novel toxin possesses a metalloprotease domain similar to BoNT/B but cleaves VAMP at a unique site (8). These discoveries, in addition to the well-documented prophage-borne BoNT/C and /D in Group III *C. botulinum* (25) and plasmid-borne BoNT/G in Group IV (26), strongly imply a role for horizontal gene transfer in shaping the modern species. Movement of BoNT-encoding plasmids into nontoxigenic clostridial strains, particularly species with more resistant phenotypes, would pose real threats to health and safety, especially given the wide prevalence of clostridia in the environment and in the mammalian gastrointestinal tract.

Despite their large natural reservoir, the functions of *C. botulinum* and BoNTs in the environment are unknown. *C. botulinum* spores are ubiquitous in soils, dust, and marine sediment, and the species can persist in the intestines of healthy animals (27). When vertebrates die and their carcasses decompose, the associated microbes and their community dynamics can change dramatically, facilitating shifts in nutrient cycling and rapid ecological succession (28). In communities like the intestinal and soil microbiomes, conjugation serves as an efficient way to transfer beneficial genes, as exemplified by widespread antibiotic resistance (29). The acquisition and formation of BoNTs may give clostridia a selective advantage by triggering vertebrate death and decomposition, thus generating a nutrient-rich substrate for population growth and perpetuation of the species (30).

Although long suggested (31), the ability of BoNT plasmids and their toxin genes to be mobilized remained unclear for many years. It was not until 2010 that experiments by Marshall *et al.* first demonstrated the movement of three BoNT-encoding plasmids between strains of *C. botulinum*, with data suggesting conjugation as the mechanism (32). In the initial study, the plasmid-borne and/or chromosomal genes required for transfer were not identified (32). Furthermore, the recipient strains contained a copy of Tn*916*; this transposon was artificially introduced to *C. botulinum* strains from an enterococcal host in our laboratory and served as a suitable positive selection marker (Tc^R) for transconjugants (32, 33). As Tn*916* is a conjugative transposon that can mobilize non-conjugative plasmids (34, 35), its involvement in plasmid mobilization could not be excluded in the earlier study (32). The mobility of Tn*916* was also problematic on the rare occasions that the transposon excised from the recipient and integrated into the donor, conferring the same antibiotic resistance phenotype as plasmid conjugation (Em^RTc^R) (32). In order to further investigate whether BoNT-encoding plasmids are conjugative, this study has recapitulated plasmid transfer in the absence of Tn*916*. The work has additionally examined the transfer of a BoNT-encoding plasmid to a wider range of clostridial recipient strains than previously shown.

Methods

Biosafety and biosecurity

Clostridium botulinum and BoNTs are classified as Tier 1 Category A Select Agents, the highest security group of biological agents. The Johnson laboratory and personnel are registered with the Federal Select Agent Program for research involving BoNTs and BoNT-producing strains of clostridia. The research program, procedures, documentation, security, and facilities are closely monitored by the University of Wisconsin-Madison Biosecurity Task Force, University of Wisconsin-Madison Office of Biological Safety, University of Wisconsin Select Agent Program, and the Centers for Disease Control and Prevention. All personnel continually undergo suitability assessments and rigorous and biosafety training, including biosafety level 3 (BSL3) or BSL2 and select agent practices, before participating in laboratory studies involving BoNTs and neurotoxigenic *C. botulinum*. All plasmid transfer experiments were conducted using plasmid pCLJ on which the major *bont* gene had been genetically inactivated by ClosTron (32). The second plasmid-borne *bont* gene produces the minor toxin BoNT/A4, which has an LD_{50} greater than 100 ng/kg (36, 37). As the toxicity is above this threshold, creating new strains of bacteria that express BoNT/A4 is not designated as a restricted experiment/major action according to the Federal Select Agent Regulations and the *NIH Guidelines*.

Bacterial strains and culture conditions

Clostridial strains were maintained at 37°C on the following media: TPGY (50 g/l trypticase peptone, 5 g/l Bacto peptone, 4 g/l dextrose, 20 g/l yeast extract, 1 g/l cysteine-HCl, pH 7.4), TPM (20 g/l casein hydrolysate [NZ Case TT; Kerry Bio-Science, Beloit, WI], 10 g/l yeast extract, 5 g/l glucose, pH 7.2), TYG (rich medium; 30 g/l Bacto tryptone, 20 g/l yeast extract, 1 g/l sodium thioglycolate), and MI (minimal medium) (38). Auxotrophs were screened

on MI with the addition of 2 g/l lysine. Solid media for mating contained 4% agar; all other plates were prepared with 1.5% agar. Frozen stocks consisted of TPGY cultures supplemented with 20% glycerol and stored at -80°C. Erythromycin was used at 50 μg/ml where appropriate. All chemicals and media components were purchased from Becton Dickinson Microbiology Systems (Sparks, MD) and Sigma-Aldrich (St. Louis, MO) unless otherwise noted.

Cultures were grown under anaerobic conditions. Glass culture tubes were flushed with nitrogen gas and sealed with butyl rubber stoppers (Bellco Glass, Vineland, NJ) before sterilizing. All culture manipulations were performed in an anaerobic chamber (Forma Anaerobic System, Marietta, OH) with an initial gas mixture of 80% N_2, 10% CO_2, and 10% H_2. Resazurin was added to solid media at 2 μg/ml, and agar plates were prereduced by overnight incubation in the anaerobic chamber.

Creation of an auxotrophic plasmid donor strain

C. botulinum strain 657Ba, containing the dual-BoNT-encoding plasmid pCLJ, served as the donor strain in this study. Prior work in our laboratory utilized ClosTron technology (39) to insertionally inactivate the *bont/b* gene on pCLJ, conferring erythromycin resistance on the strain (32). The Em^R parent strain was subsequently chemically mutagenized with 1 mg/ml *N*-methyl-*N*'-nitro-*N*-nitrosoguanidine (MNNG; TCI America, Portland, OR) in a protocol adapted from the literature (40, 41). Cultures were revived from frozen stock, subcultured once, and grown to mid-log phase. Cells were collected by centrifugation, washed once with citrate buffer (100 mM citric acid – sodium citrate, pH 5.5) and resuspended in the same buffer. MNNG in citrate buffer was added to achieve a final concentration of 1 mg/ml. The cells were incubated in a 37°C water bath for 15 minutes. MNNG was then quenched with the addition of 0.5 ml cold sodium phosphate buffer (100 mM, pH 7). Cells were harvested by centrifugation, washed twice with

phosphate buffer, and resuspended in 0.5 ml buffer. The entire volume was then added to fresh TPGY broth and allowed to outgrow overnight at 37°C. Cultures were diluted and plated on TYG agar for isolated colonies. Colonies that grew on rich medium were picked with sterile toothpicks and replica plated on minimal media to identify auxotrophs. An isolate that grew on TYG and MI + lysine but failed to grow on MI was restreaked for purification and chosen for further analysis. After confirming that this isolate retained pCLJ, remained resistant to erythromycin, and suffered no growth defects in rich medium, clonal isolates of the mutant strain were utilized as a plasmid donor in all mating experiments described below (EMN053-EMN056, Table 1).

Plasmid transfer

Donor and recipient strains were revived from frozen stock, subcultured twice, and grown to $OD_{600} \approx 0.8$. In order to enumerate donors and recipients, 200 µl of each culture was spotted individually on TYG with 4% agar. To assess plasmid transfer, equal volumes (200 µl) of donor and recipient were spotted together on TYG with 4% agar. All agar plates were allowed to incubate at 37°C for 18 h. Bacterial growth was then washed with 3 ml 1 × PBS, and the suspensions were serially diluted in 1 × PBS. Dilutions of donor and recipient controls were plated on TYG with erythromycin and on MI, respectively, to determine CFU/ml. Aliquots of donor suspensions (100 µl) were spread on minimal media to test for reversion to prototrophy. Likewise, aliquots of recipient suspensions (100 µl) were spread on TYG with erythromycin to account for spontaneous mutation. Mating mixtures were diluted and plated on MI supplemented with erythromycin to enumerate transconjugants. Transfer frequency was calculated by dividing transconjugant CFU/ml by the larger of the donor or recipient CFU/ml.

Analysis of transconjugant strains

In order to verify that the observed colonies were true transconjugants (recipient strains that obtained the donor plasmid), a variety of screens were performed. Spontaneous Em^R mutants were exceedingly uncommon, and recipient strains were often visually different from the plasmid donor. Thus, colony morphology on MI + Em was typically sufficient to differentiate transconjugants from revertants. For mating pairs in which donor and recipient strains were morphologically similar, a marker gene characteristic of the recipient was amplified by PCR. Putative transconjugant colonies were picked with sterile toothpicks, swirled in 200 µl sterile water, and microwaved for 1 minute. One microliter of this template DNA was dispensed directly into *Taq* 2X Master Mixes (New England Biolabs, Ipswich, MA) and marker genes amplified using appropriate primers (Table 1).

The presence of pCLJ was also confirmed in representative transconjugants by pulsed-field gel electrophoresis (PFGE). Strains were revived from frozen stock, grown anaerobically overnight at 37°C, and subcultured into 10 ml TPGY broth. Growth of the subcultures was then monitored until their OD_{600} reached 1.2 (ThermoFisher Spectronic 200, Waltham, MA), at which point 1 ml formaldehyde was added and the cultures were placed on ice. PFGE plugs were prepared as previously described (42). One plug from each sample was processed without restriction enzyme digest, and a second was digested with XhoI (New England Biolabs, Ipswich, MA), a rare cutting enzyme that linearizes pCLJ. DNA was separated by electrophoresis in a clamped homogeneous electric field system at 6 V/cm, 12°C, 1-26 s pulse time for 24 h (CHEF-DRII; Bio-Rad, Hercules, CA). Samples were stained with 1 µg/ml ethidium bromide for 20 min, washed in sterile water 5 × 30 min to destain, and photographed using a Fotodyne/FOTO/Analyst FX imaging system and software (Harland, WI). If necessary, image brightness and contrast were equally adjusted using Adobe Photoshop CC software.

To prepare PFGE samples for Southern hybridization, gels were transferred to a Nytran SuPerCharge membrane (GE Healthcare Life Sciences, Marlborough, MA) overnight by downward capillary transfer (43). Membranes were wet in distilled water for 15 min, washed in transfer buffer (0.4 M NaOH, 1.5 M NaCl) for 15 min, and transferred using the GE Healthcare Life Sciences Turboblotter stack (Marlborough, MA) for approximately 16 h. Following transfer, the membranes were neutralized for 15 min in 2 M Tris-HCl, pH 7.0. They were then rinsed for 15 min in 2 × SSC (3 M NaCl, 0.3 M sodium citrate) and dried under vacuum for 1 h at 80°C. Membranes were stored at 4°C until hybridization. Hybridization probes were amplified from a recombinant plasmid copy of *bont/a4* (36) by PCR with Phusion High Fidelity Master Mix (New England Biolabs, Ipswich, MA) using the three A4 primer pairs listed in Table 1. Gene fragments were purified from agarose gels using Qiagen's MinElute kit (Germantown, MD) and radiolabeled with α-^{32}P ATP using the Megaprime DNA labeling system (GE Healthcare Life Sciences, Marlborough, MA). Membranes were individually pre-washed in a solution of 50 mM Tris-HCl pH 8.0, 1 M NaCl, 1 mM EDTA, and 0.1% SDS for 2.5 h at 60°C, then rinsed with 5 × SSPE. Each membrane was incubated for 2.5 h at 42°C in 6 × Denhardt's solution, 5 × SSPE, 50% formamide, 0.5% SDS, 100 μg/ml herring sperm DNA (Promega, Madison, WI). A mixture of the three radiolabeled probes was then added to each bottle at approximately 5×10^7 cpm/ml and hybridization carried out for 17 h at 42°C. Membranes were washed (a) twice with 1 × SSPE and 0.1% SDS for 5 min at RT, (b) once with 1 × SSPE and 0.1% SDS for 10 min at 42°C, and (c) once with 0.1 × SSPE and 0.1% SDS for 10 min at 42°C. Autoradiography took place at -80°C overnight using blue x-ray film (Phenix Research Products, Candler, NC) and a BioMax intensifying screen (Eastman Kodak, Rochester, NY).

Transconjugant strains were analyzed at the protein level by SDS-PAGE and Western blotting. Strains were revived from frozen stock and cultured in TPM at 37°C for 48 h.

Subsamples of the whole cultures were removed anaerobically and centrifuged 5 min to pellet cells. A portion of the supernatant was treated with 50 μg/ml TPCK-treated trypsin (Worthington Biochemical Corp., Lakewood, NJ) for 30 min at 35°C. Type II-S soybean trypsin inhibitor (Sigma-Aldrich, St. Louis, MO) was then added to this portion at a final concentration of 100 μg/ml and incubated at room temperature for 10 min. All samples were denatured by adding NuPAGE SDS sample buffer (Life Technologies, Carlsbad, CA) to 1× concentration and heated to 95°C for 5 minutes. Samples were reduced with the addition of 100 mM DTT as desired. Proteins were separated by electrophoresis at 150 V through 4-12% Bis-Tris NuPAGE Novex gels in morpholineethane sulfonic acid (MES) running buffer (Life Technologies, Carlsbad, CA). The gels were stained using Instant Blue (Expedeon Inc., San Diego, CA), and various markers including the SeeBlue Plus2 Prestained Standard and the MagicMark XP Western Standard (Life Technologies, Carlsbad, CA) were used to estimate molecular weight. Gels were transferred to PVDF membranes (MilliporeSigma, Burlington, MA) using a semi-dry transfer protocol (0.4 A, 1 h). Membranes were probed for the presence of BoNT/A with polyclonal affinity-purified rabbit IgG antibodies raised in our laboratory against BoNT/A1 and a bovine anti-rabbit secondary antibody (Santa Cruz Biotechnology, Dallas, TX). Images were developed with the chemiluminescent PhosphaGLO alkaline phosphatase substrate (KPL, Gaithersburg, MD) and photographed as above.

Mouse bioassay

Toxicity of culture supernatants was determined by mouse bioassay as previously described (44, 45). Briefly, culture supernatants from parent strains and transconjugant derivatives grown at 37°C for 48 h were passed through a 0.2 μm filter. To evaluate neutralization, monovalent antitoxin produced in our lab was incubated with culture supernatant

at ambient temperature for 60 min. All samples were diluted three-fold in 0.03 M sodium phosphate buffer (pH 6.3) with 0.2% gelatin (GelPhos). Two mice per sample were injected intraperitoneally with 0.5 ml of the GelPhos dilution (i.e. approximately 0.167 ml of the culture supernatant) and observed for signs of botulism for 4 days (46). The mouse research was conducted according to protocols approved by the CDC Institutional Animal Care and Use Committee.

Statistical analysis

Selected mating assays were compared using an unpaired Student's *t*-test. The difference in transfer frequencies between two recipient strains is described as statistically significant if $P \leq 0.05$.

Results

MNNG mutagenesis generates stable C. botulinum *auxotrophs*

Prior experiments demonstrated the transfer of pCLJ to *C. botulinum* strains containing Tn*916*, a conjugative transposon that confers tetracycline resistance (32). In order to eliminate the need for a second selective marker in the recipient strain and to expand the range of potential recipients, an auxotrophic donor strain was created. After multiple attempts to chemically mutagenize *C. botulinum* strain 657Ba (pCLJ-Erm) (Table 1) with *N*-methyl-*N*'-nitro-*N*-nitrosoguanidine (MNNG), a single lysine auxotroph was isolated from approximately 200 screened colonies. Lysine is nonessential for Group I *C. botulinum* and was chosen for this screen based on an auxotrophy identified in a prior mutagenesis experiment (33). The current mutant has no apparent growth defects in rich medium; it grows on minimal medium (MI) (38) supplemented with lysine but not on unsupplemented MI (Figure 1). The auxotrophy is stable over repeated passages and reverts at low frequency (less than 10^{-10}).

Table 1. Bacterial strains and oligonucleotide primers. All strains were grown under standard anaerobic culture conditions. Where possible, a source describing the isolation or characterization of the strain is given. Additional information is available upon request.

Name	Relevant Characteristics	Reference
C. botulinum 657Ba	*bont*/*b5* and *bont*/*a4* on pCLJ	(19)
C. botulinum 657Ba (pCLJ-Erm)	*bont*/*b5*::*ermB* and *bont*/*a4* on pCLJ	(32)
EMN053-EMN056	*bont*/*b5*::*ermB* and *bont/a4* on pCLJ, lys^-	This study
C. botulinum Hall A-*hyper*	*bont*/*a1* on chromosome	(47)
C. botulinum Hall A-*hyper* Tn*916*	Tc^R, *bont*/*a1* on chromosome	(32)
C. botulinum 62A	*bont*/*a1* on chromosome	(48)
C. botulinum LNT01	Tc^R, nontoxigenic	(49)
C. botulinum Marsh 51B	*bont*/*c* on prophage	EAJ*
C. sporogenes C	Nontoxigenic	EAJ*
C. sporogenes 4439	Nontoxigenic	EAJ*
C. sporogenes WR5	Nontoxigenic	EAJ*
C. sporogenes 19404	Nontoxigenic	(50)
C. sporogenes PA3679	Nontoxigenic	(51)
C. butyricum 13983	Nontoxigenic	EAJ*
C. butyricum 5520	*bont/e* on chromosome	(52)
A1-500F	GCTTTGGACATGAAGTTTTGAATC	This study
A1-1825R	GTTCTACCCAGCCTAAAAACATAG	This study
A4-535F	GGTTATGGTTCTACTCAATACATTAG	This study
A4-809R	CCCCCAAATGTTATAAGTTCCTC	This study
A4-1030F	CTGTTAACAGAGATTTACACAGAGG	This study
A4-1824R	CCAATTAACAAACGTAACTGCCTC	This study
A4-2001F	TGTACCAGAGATTGCGCTACCTG	This study
A4-2674R	CTATTAAATCATCATCTTTATATACTATGCTC	This study

*Strain names given as in laboratory records. Provenance unknown.

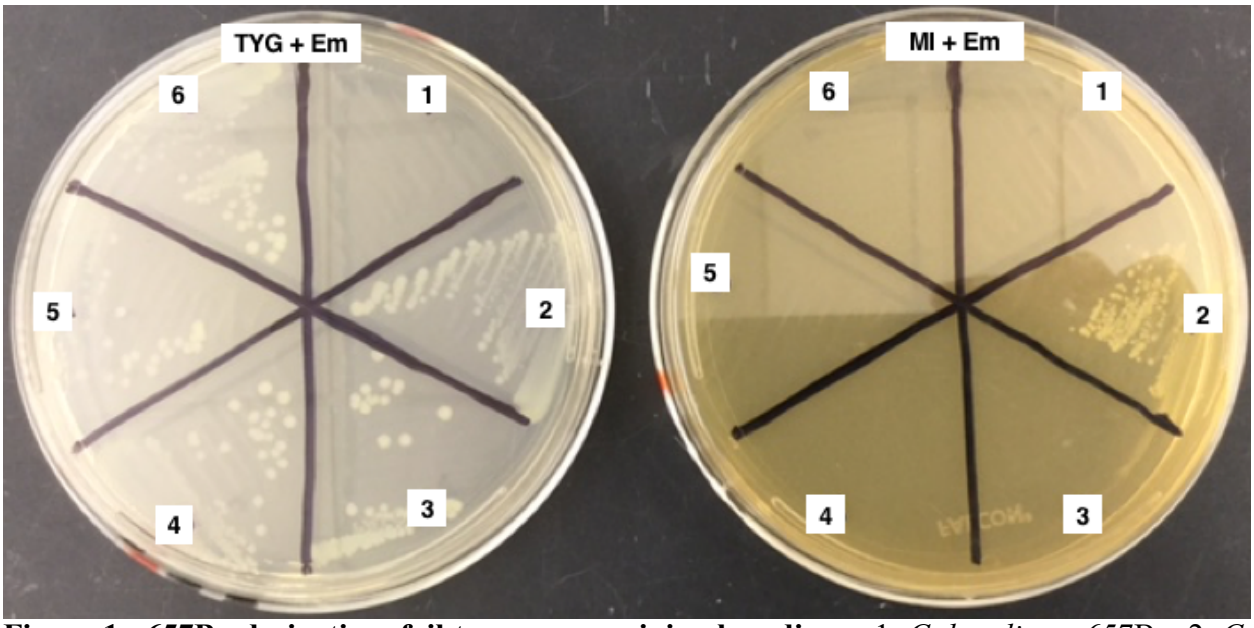


Figure 1. 657Ba derivatives fail to grow on minimal medium. 1, *C. botulinum* 657Ba; 2, *C. botulinum* 657Ba (pCLJ-Erm); 3-6, EMN053-056. See Table 1 for additional strain information. At left, rich medium with 50 µg/ml erythromycin. At right, minimal medium with 50 µg/ml erythromycin. Auxotrophs can be restored to wild-type growth levels with the addition of 2 g/l lysine.

*Plasmid transfer does not require Tn*916

As the lysine auxotrophs retained the Em^R phenotype of the parent strain, they were used to demonstrate that pCLJ could be conjugated to unmarked recipients in a Tn*916*-free mating scheme (Table 1). Transfer frequencies for pCLJ ranged as high as 10^{-6} transconjugants per donor cell (Table 2). To confirm that the observed transconjugants were not revertant donor colonies, a subset was initially analyzed by PCR. One representative experiment is shown, in which 100% of putative transconjugants possessed the *bont*/*a1* gene indicative of the recipient background (Figure 2). Certain unmarked recipient strains, namely Hall A-*hyper* and 62A, have Tn*916* derivatives generated for use in prior experiments in our laboratory. In order to evaluate the transposon's role in plasmid transfer, these derivatives (strains Hall A-*hyper*/Tn*916* and LNT01, respectively) were used as recipients in selected auxotrophic mating experiments (Table 1). In these experiments, Tn*916* was present on the chromosome of recipients but was not used

as a selectable marker. The transfer frequency of pCLJ to recipients carrying Tn*916* did not significantly differ from the transfer frequency to their parent strains, suggesting that the transposon was nonessential for plasmid mobilization (Table 2, Student's *t*-test: Hall A-*hyper* vs. Hall A-*hyper*/Tn*916*, $P = 0.15$; 62A vs. LNT01, $P = 0.1153$).

Table 2. pCLJ is capable of conjugative transfer into a variety of clostridial strains. pCLJ, a 270 kb plasmid encoding two BoNTs, was transferred from *C. botulinum* 657Ba to selected recipient strains. Plasmid transfer was conducted in overnight matings on solid media. Transfer frequencies were calculated by dividing transconjugant CFU/ml by the larger of the donor or recipient CFU/ml. Minimum $n = 3$, ± s.d.

Recipient Species	Strain	*C. botulinum* Group	BoNT Serotype	Mean Transfer Frequency
C. botulinum	Hall A-*hyper*	I	A	$2.0 \times 10^{-6} \pm 2.8 \times 10^{-6}$
	Hall A-*hyper*/Tn*916*	I	A	$6.0 \times 10^{-6} \pm 6.9 \times 10^{-6}$
	62A	I	A	$3.9 \times 10^{-9} \pm 6.5 \times 10^{-9}$
	LNT01	I	*	$9.9 \times 10^{-11} \pm 1.6 \times 10^{-10}$
	Marsh 51B	III	C	$1.3 \times 10^{-13} \pm 1.5 \times 10^{-13}$
C. sporogenes	C			$1.1 \times 10^{-10} \pm 1.9 \times 10^{-10}$
	4439			$1.1 \times 10^{-10} \pm 1.9 \times 10^{-10}$
	WR5			$1.3 \times 10^{-10} \pm 2.1 \times 10^{-10}$
	19404			$1.5 \times 10^{-13} \pm 1.2 \times 10^{-13}$
	PA3679			$3.7 \times 10^{-14} \pm 4.8 \times 10^{-14}$
C. butyricum	13983			$1.4 \times 10^{-10} \pm 8.4 \times 10^{-11}$
	5520	VI	E	$2.4 \times 10^{-10} \pm 3.2 \times 10^{-10}$

*Nontoxigenic Tn*916* mutant derived from strain 62A.

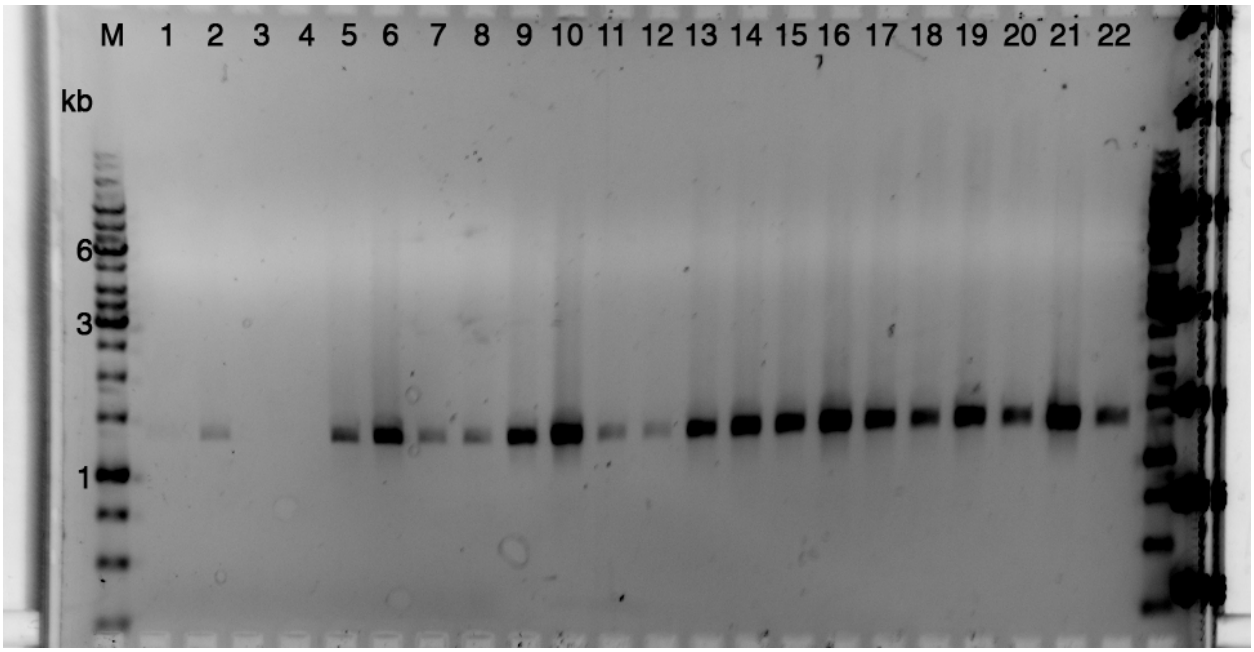


Figure 2. Colony PCR confirms transconjugant genotype. A marker gene characteristic of the recipient strain (*bont/a1*) was amplified by colony PCR using the A1 primer pair in Table 1 and analyzed by agarose gel electrophoresis. Lanes 1-2, recipient strains; lanes 3-4, pCLJ donor strains; lanes 5-22, selected transconjugants. M, O'GeneRuler 1 kb DNA ladder (Thermo Scientific, Waltham, MA). A full-length gel is presented in Supplementary Figure S1.

BoNT-encoding plasmids are transferred to various clostridial species

A diverse panel of Group I *C. botulinum* strains received pCLJ in conjugative matings (Table 2). pCLJ was also capable of transfer into non-*botulinum* species. *C. sporogenes* recipients had lower transfer frequencies than *C. botulinum* recipients. *C. butyricum* strains were also suitable recipients, with transfer frequencies around 10^{-10} transconjugants per donor (Table 2). Although the incompatibility group of pCLJ is not known, the complete nucleotide sequence of *C. botulinum* strain 657Ba possesses two extrachromosomal replicons—the 270 kb plasmid described in this work, and a second cryptic plasmid of approximately 10 kb (NCBI Reference Sequence NC_012657.1). The smaller plasmid has not been purified or functionally characterized, nor have we monitored its stability or segregation in our laboratory isolates. Attempts to transfer pCLJ to a recipient strain with a comparably large plasmid were unsuccessful, as matings between the pCLJ donor and *C. botulinum* strain Okra B, which

contains a BoNT-encoding plasmid of ~149 kb, did not yield transconjugant colonies. This suggests that the large plasmids of Group I *C. botulinum* are mutually exclusive, and available sequence information agrees that bivalent strains do not have more than one large BoNT-encoding plasmid (53).

pCLJ is stable in transconjugant strains and produces active BoNT/A4

As all transconjugants were recovered on the basis of plasmid-encoded antibiotic resistance, pCLJ was functional upon transfer to recipient strains. In the absence of antibiotic pressure and under standard culture conditions, the plasmid was maintained for at least five passages (data not shown). pCLJ was fully transferred to recipient strains, as evidenced by the appearance of a ~270 kb band in PFGE samples of representative transconjugants (Figure 3). Moreover, the ~270 kb band consistently hybridized with radiolabeled probes against pCLJ's active neurotoxin gene, *bont/a4* (Figure 4). Note that the chromosomal *bont/a1* genes of certain transconjugant strains were also detected by the *bont/a4* probe; the high sequence identity between the two toxins prevented us from designing a truly plasmid-specific *bont/a* probe (Figure 4). At the protein level, the cell lysates of parent and transconjugant strains had similar profiles, suggesting that the proteome was not globally altered by the presence of pCLJ (Figure 5a). Plasmid genes were expressed in the transconjugant, as BoNT/A4 was detected in *C. sporogenes* (pCLJ) culture supernatants (Figure 5b; Supplementary Figure S5). As determined by immunoblot at 48 h, the BoNT was primarily in the supernatant of cultures sampled, and it was proteolytically nicked as evidenced by the appearance of two bands following reduction of the disulfide bond (Figure 5b). Culture supernatants of plasmid donor strains and *C. sporogenes* (pCLJ) transconjugant strains, but not of the nontoxigenic *C. sporogenes* PA3679 recipient

strain, were lethal in the mouse bioassay. Mice were protected by neutralization with monovalent antibody against BoNT/A1 (Table 3).

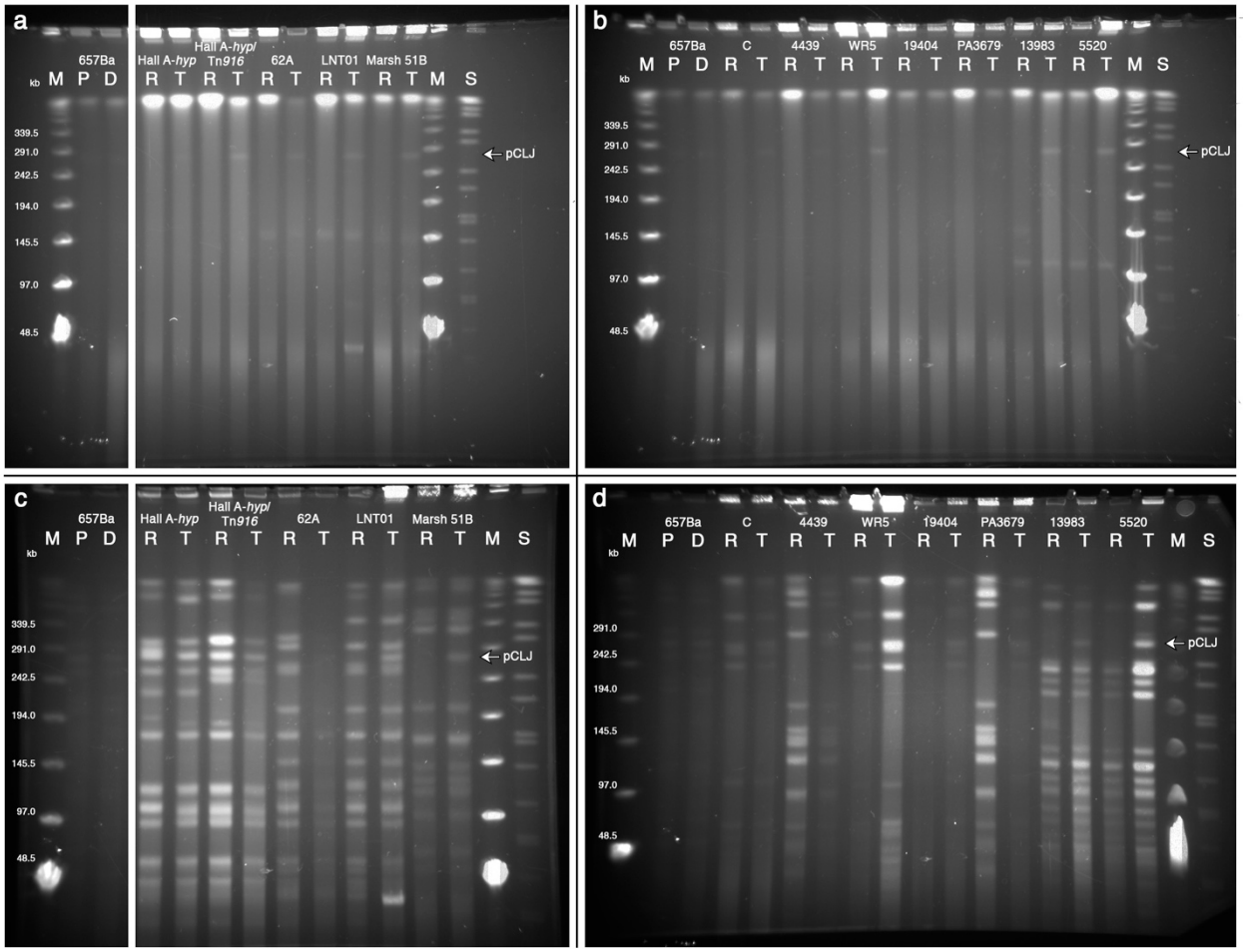


Figure 3. Full-length pCLJ is maintained in transconjugant strains. Matings were conducted as described in the text. PFGE plugs were prepared from the given strains and electrophoresed undigested (a-b) and following XhoI digest (c-d). pCLJ, at ~270 kb, is marked with an arrow. P, *C. botulinum* 657Ba (pCLJ-Erm) parent strain; D, pCLJ auxotrophic donor strain; R, recipient strains; T, transconjugant strains; M, Lambda PFG Ladder; S, *Salmonella enterica* serotype Braenderup strain H9812, XbaI digest (54). All full-length gels are presented in Supplementary Figure S2.

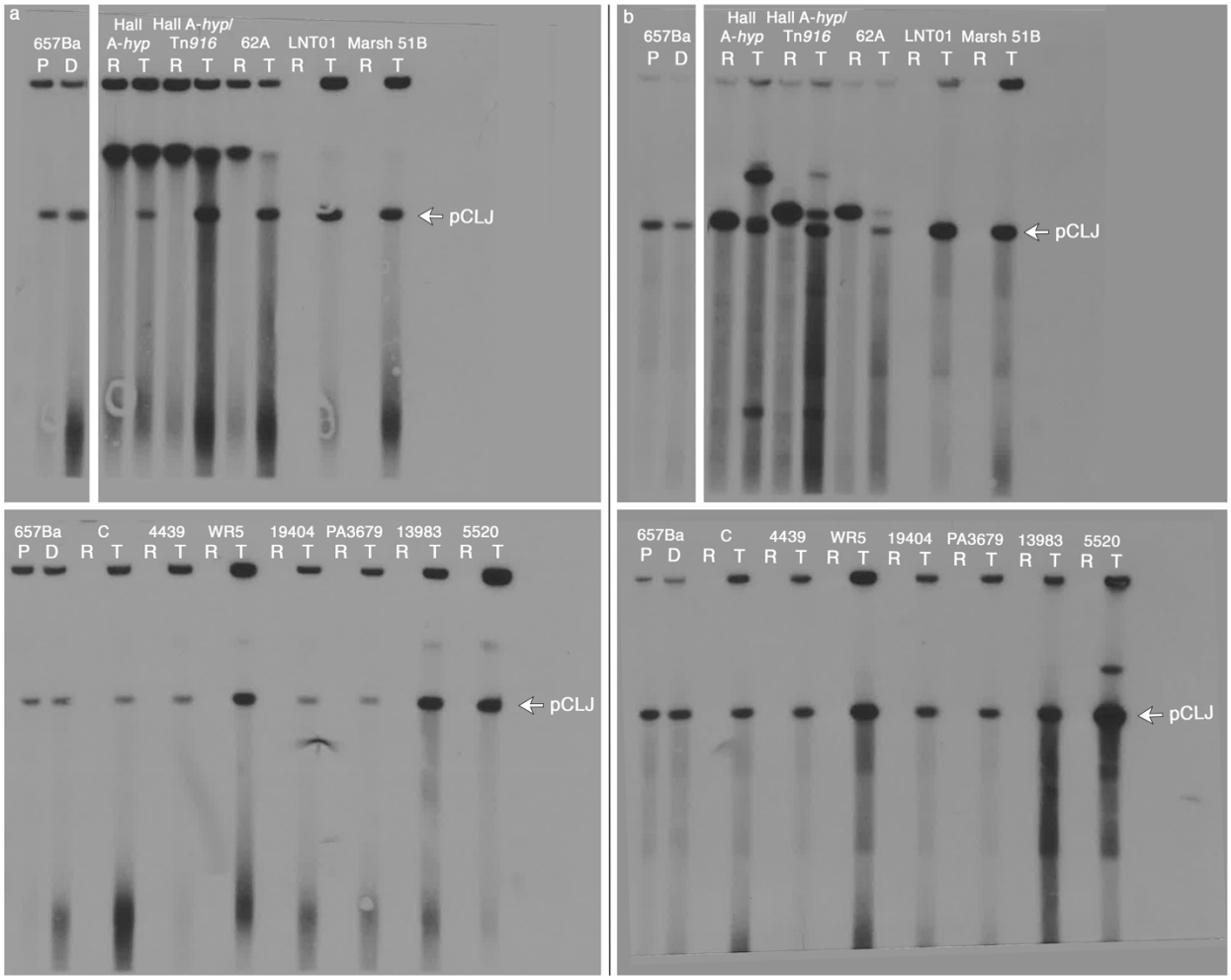


Figure 4. *bont/a4* remains associated with pCLJ in transconjugant strains. Undigested PFGE samples (a) and XhoI-digested samples (b) were transferred to nylon membranes and hybridized to *bont/a4* probes. pCLJ, at ~270 kb, is marked with an arrow. P, *C. botulinum* 657Ba (pCLJ-Erm) parent strain; D, pCLJ auxotrophic donor strain; R, recipient strains; T, transconjugant strains. Uncropped blots are presented in Supplementary Figure S3.

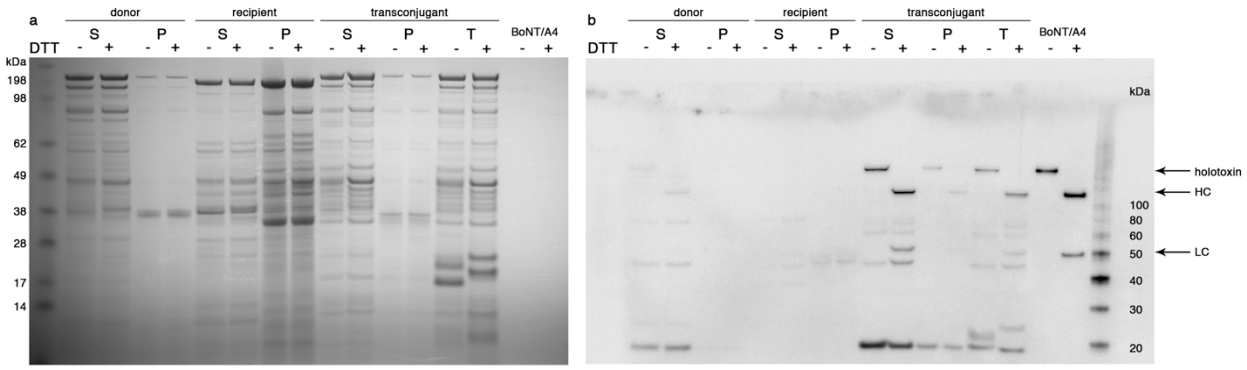


Figure 5. Transconjugant strains are converted to toxigenicity. Samples of donor strain EMN053, recipient strain *C. sporogenes* PA3679, and transconjugant strain *C. sporogenes* PA3679 (pCLJ) were collected at 48 h and prepared as described in the text. S, supernatant; P, pellet; T, trypsinized supernatant. Gels were (a) stained to visualize total proteins or (b) immunoblotted for the presence of BoNT/A. Purified BoNT/A4 was used as a control. Samples were reduced with 100 mM DTT as indicated. Full-length images are presented in Supplementary Figure S4.

Table 3. Transconjugants produce active BoNT/A4. Two mice per sample were injected with culture supernatants (treated as described) and observed for signs of botulism. Numerators indicate mice surviving on a given day.

Sample	Treatment	Day 1	Day 2	Day 3
EMN053 (pCLJ donor)		2/2	2/2	0/2
C. sporogenes 4439 (pCLJ)		2/2	0/2	
C. sporogenes C (pCLJ)		2/2	0/2	
C. sporogenes PA3679		2/2	2/2	2/2
C. sporogenes PA3679 (pCLJ)		2/2	0/2	
C. sporogenes PA3679 (pCLJ)	Incubated with antitoxin	2/2	2/2	2/2

Discussion

The functional and molecular aspects of BoNT-encoding plasmids, despite their likely role in the horizontal transfer of neurotoxin genes, remain largely enigmatic. Prior to this work, BoNT-encoding plasmids had been experimentally transferred, but only to *C. botulinum* recipients containing Tn*916*. Conjugation was the likely mechanism, as DNA transfer required cell-cell contact, was not mediated by donor culture supernatants, and was not prevented by DNaseI treatment (32). Here, we have excluded Tn*916* from mating pairs to demonstrate that the BoNT-encoding plasmid pCLJ is not mobilized via transposon. In addition, we have extended the host range of pCLJ to non-*botulinum* species of *Clostridium*, supporting the hypothesis that horizontal gene transfer contributes to the diversity, toxigenicity, and function of the genus.

Transfer of BoNT plasmids is a low frequency event under our experimental conditions, and may well be even lower in natural environments. Nevertheless, given the extreme potency of BoNTs, their movement into new strains is cause for concern. Conjugation of BoNT plasmids into formerly nontoxigenic species poses threats to animal and human health and food safety, as these species have different disease tropisms and resistance phenotypes than *C. botulinum*. Strains producing BoNTs from the chromosome were typically amenable to plasmid transfer (Table 2). Recipient strains producing BoNT from their own plasmid, e.g. Okra B, did not yield appreciable pCLJ transconjugants, indicating that the toxin plasmids in Group I *C. botulinum* may be incompatible. It is possible that pCLJ is capable of conjugative transfer to plasmid-bearing strains outside Group I, but this was not determined due to the limitations on media and culture conditions in the current assay. Multiple plasmids of varying sizes coexist in Group III *C. botulinum* strains, but to our knowledge, only one plasmid lineage per cell carries the *bont* gene (25, 55). In species that are already toxigenic, the introduction of a new BoNT due to

conjugative plasmid transfer could make the strain multivalent, leading to difficulties in detection, diagnosis, and treatment. Conversely, experimental conjugation of plasmid-borne toxins to heterologous hosts (e.g. Figure 5) could aid in BoNT production and purification.

pCLJ transferred with the highest frequency to *C. botulinum* Hall A-*hyper*, a strain identified for maximal BoNT/A production (Table 2). Hall A-*hyper* is a unique lab-adapted strain; it is missing cell surface and membrane proteins that are present in reference *C. botulinum* genomes (56). The loss of these features may allow closer cell-cell contact between mating pairs, perhaps enabling pCLJ to transfer more easily to Hall A-*hyper* recipients. Physical barriers are only one impediment to conjugation between bacterial strains, however, and there must surely be other factors that contribute to the observed discrepancies in transfer frequency across the clostridia. The genetic diversity between donor and recipient strains did not show a clear correlation to the transfer frequency within the mating pair (Table 2).

In all transconjugants we monitored, pCLJ was maintained in at least one full-length extrachromosomal copy (Figures 3-4). In two transconjugants, we observed the appearance of a larger XhoI-generated fragment that hybridized with *bont/a4* probes (Figure 4). Further analysis is necessary to ascertain whether this signal is due to recombination of the plasmid-borne *bont/a4* gene with a chromosomal *bont* gene, incomplete restriction digest of the sample, altered plasmid mobility, or another factor. In the vast majority of transconjugants, including all *C. sporogenes* transconjugants, we did not observe the integration or recombination of the *bont/a4* gene between plasmid and chromosomal replicons. Intriguingly, sequence information from a newly identified BoNT/FA-producing strain suggests that toxin genes may have moved from plasmid to chromosome or the reverse. This mosaic toxin is encoded in a unique chromosomal locus compared to other *bont* genes, is adjacent to a “pCLJ-like” region, and is flanked by IS elements,

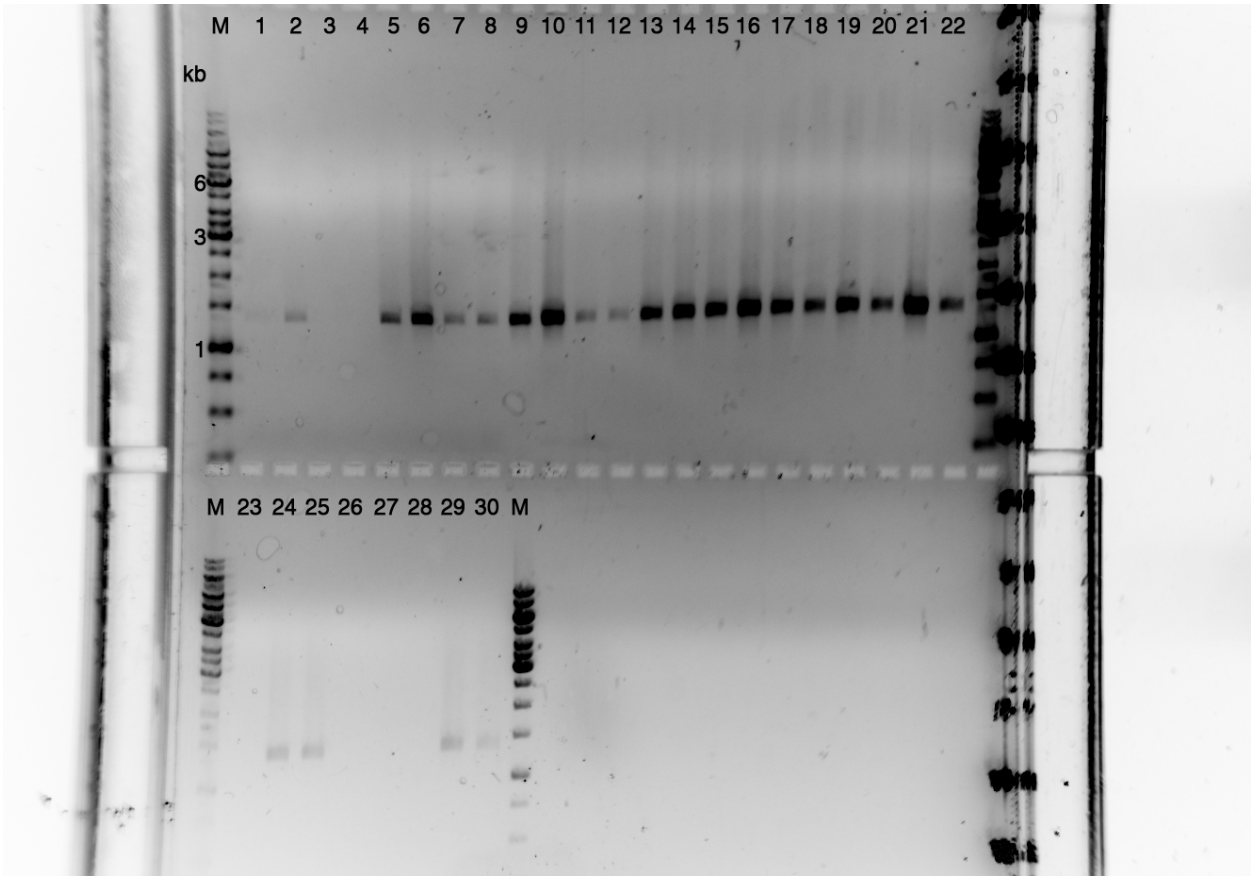
implying that the toxin cluster was moved between replicons by an unknown recombination event (57).

In gram-positive bacteria, conjugative transfer systems often bear a resemblance to type IV secretion systems, including ATPases, mating channel proteins, and coupling proteins (58). In *Clostridium*, the most well-characterized conjugation system is the *tcp* locus of *C. perfringens* toxin plasmids (22). These are typified by pCW3, a ~47 kb plasmid that confers tetracycline resistance and transfers at high frequency. Intriguingly, the *tcp* locus is similar to the conjugation locus from Tn*916* (59). The pCLJ sequence annotations do not indicate any obvious parallels to the pCW3/Tn*916* model, and accordingly the transfer frequencies reported here are several orders of magnitude lower than observed in *C. perfringens* (60). There is, however, a large region of pCLJ that contains many type II/IV secretion components in addition to an ATPase, a helicase, and a cell wall hydrolase (61). A homologous region is seen in the sequence of pCLK, a Group I *C. botulinum* plasmid close in size to pCLJ, which was also experimentally mobilized (32). The small second plasmid present in the *C. botulinum* 657Ba reference strain (NCBI Assembly GCA_000020345.1) does not contain this so-called "conjugation region" and has very little sequence homology to other clostridial plasmids, as determined via nucleotide BLAST (62). Still, it is possible that the smaller plasmid and/or genes from the donor chromosome play an accessory role in pCLJ transfer. Efforts are ongoing to determine the functions of genes in the conjugation region of pCLJ and explore their contributions to plasmid transfer in *C. botulinum*. Detailed study of the pCLJ conjugative apparatus may ultimately reveal a much-needed source of genetic tools for molecular manipulation of *C. botulinum*.

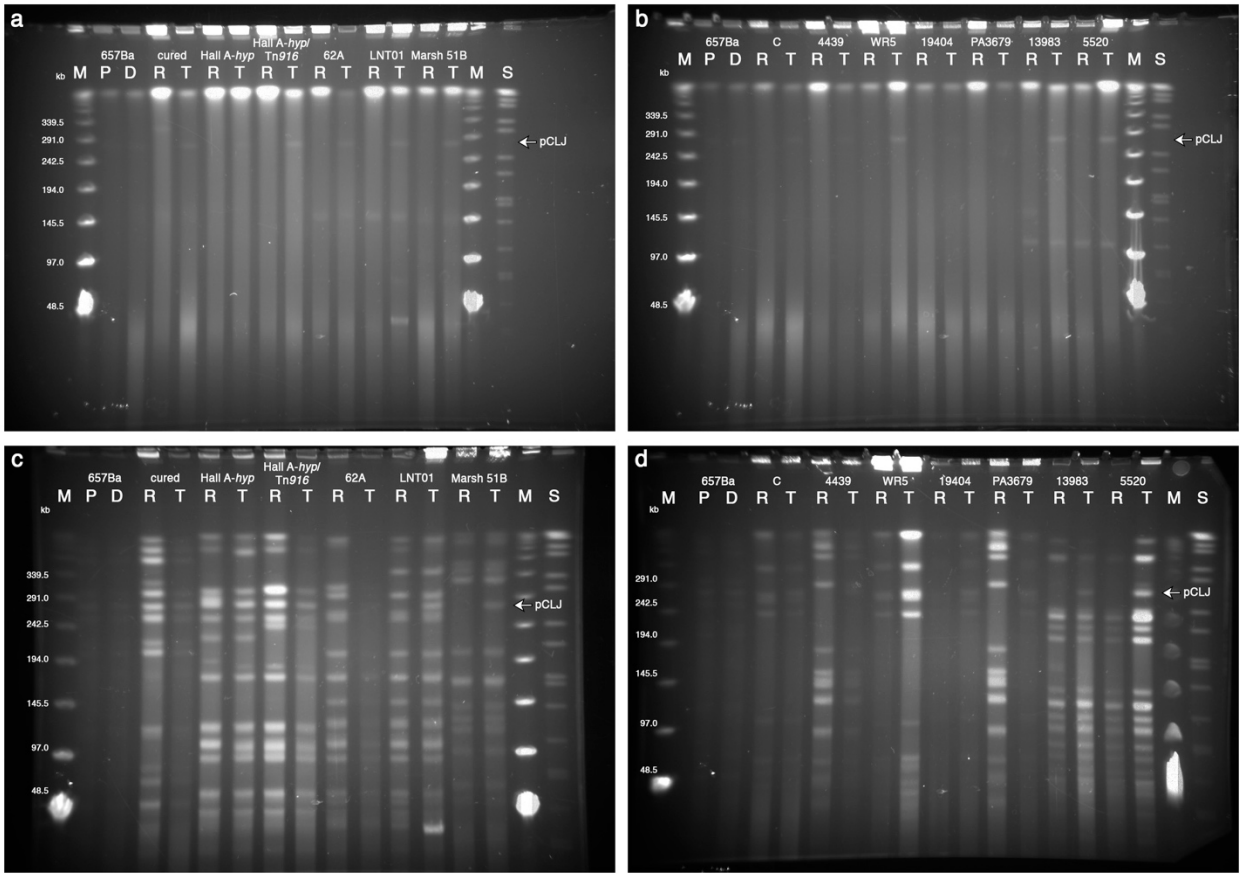
This work could also contribute to the enigmatic functions of BoNTs in nature. The neuromuscular junction has been considered the most vulnerable target of toxins leading to morbidity and mortality, and the phrenic nerve controlling breathing is among the most sensitive

neuromuscular sites (2). Considerable work has been performed on the microbe-mediated decomposition of animal and human carcasses (63, 64), but these studies have been done on animals following their death. The actual events that trigger deaths in animals are not always well-defined, but botulinum toxins are known to cause hundreds of thousands of deaths in wildlife every year (65), and it is plausible that botulinum toxins play such a role in death and ensuing animal decomposition and recycling of nutrients.

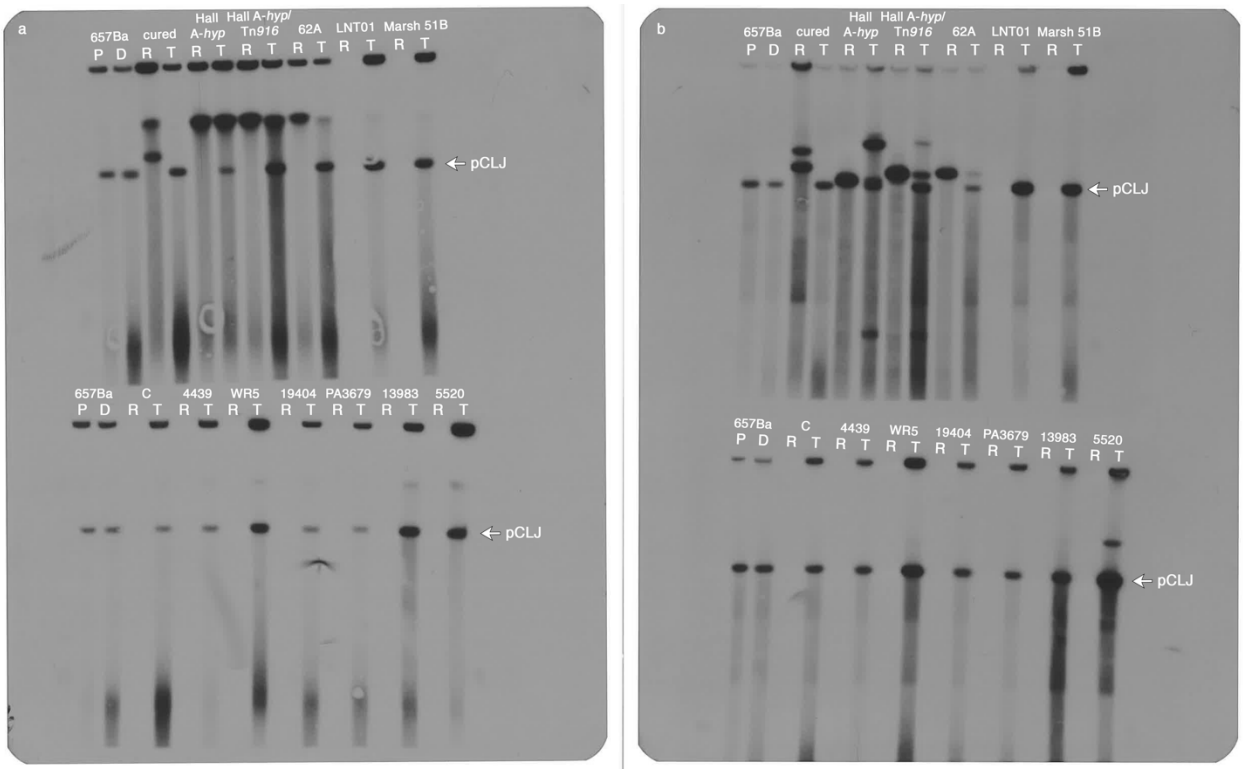
Supplementary Information



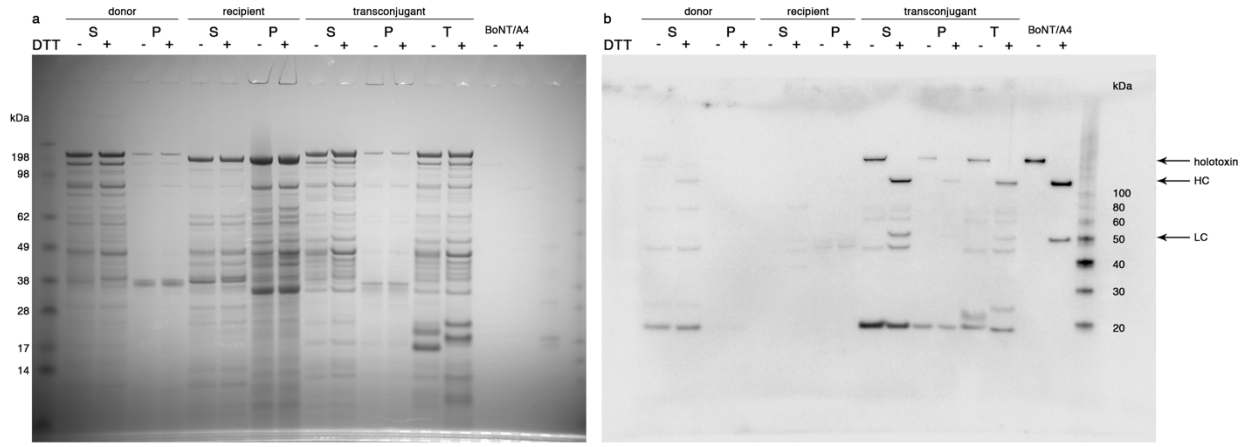
Supplementary Figure S1. Colony PCR confirms transconjugant genotype. A marker gene characteristic of the recipient strain (bont/a1) was amplified by colony PCR using the A1 primer pair in Table 1 and analyzed by agarose gel electrophoresis. Lanes 1-2, recipient strains; lanes 3-4, pCLJ donor strains; lanes 5-22, selected transconjugants. M, O'GeneRuler 1 kb DNA ladder (Thermo Scientific, Waltham, MA). Lanes 23-30 show a colony PCR screen from an independent conjugation experiment.



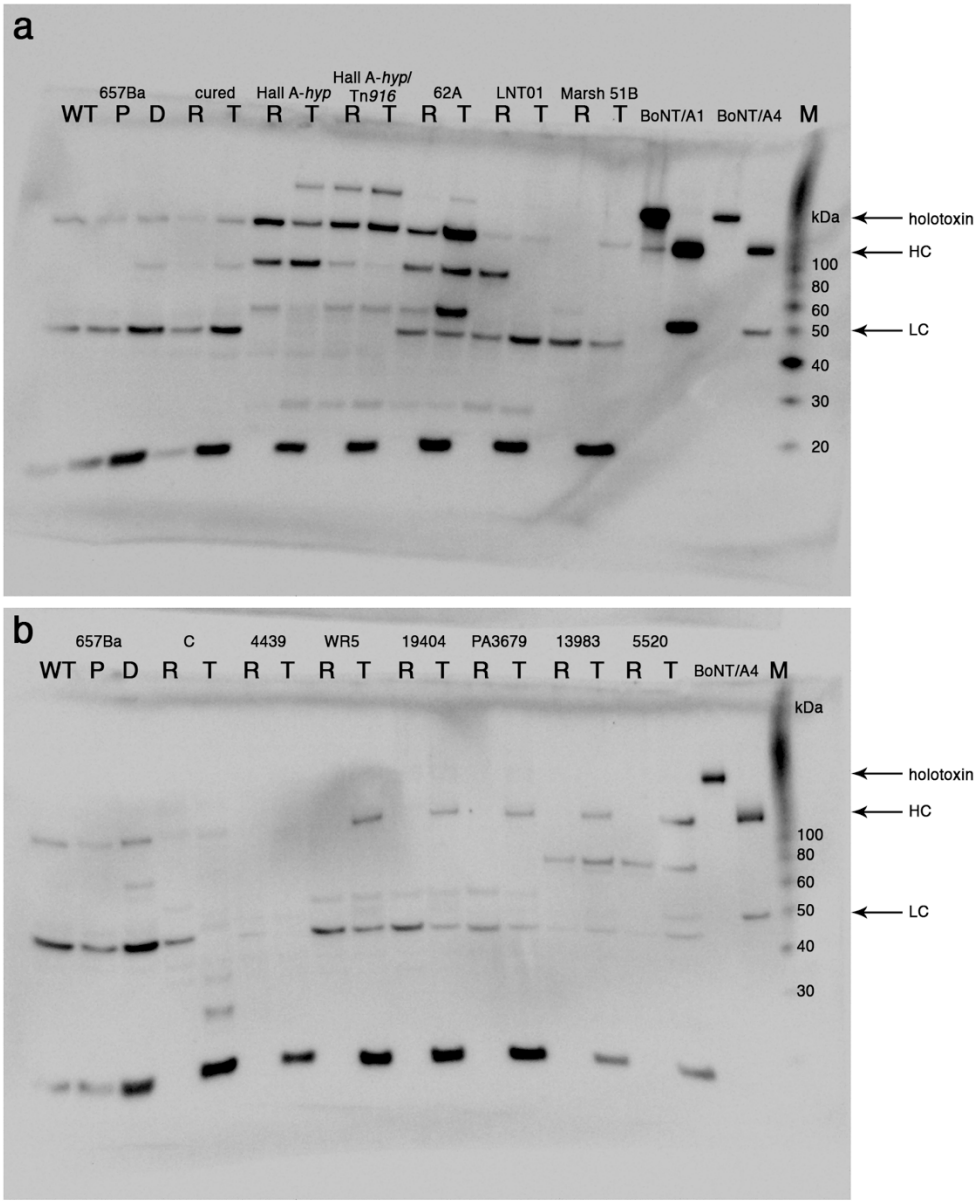
Supplementary Figure S2. Full-length pCLJ is maintained in transconjugant strains. Matings were conducted as described in the text. PFGE plugs were prepared from the given strains and electrophoresed undigested (a-b) and following XhoI digest (c-d). pCLJ, at ~270 kb, is marked with an arrow. P, *C. botulinum* 657Ba (pCLJ-Erm) parent strain; D, pCLJ auxotrophic donor strain; R, recipient strains; T, transconjugant strains; M, Lambda PFG Ladder; S, *Salmonella* XbaI digest.



Supplementary Figure S3. *bont/a4* remains associated with pCLJ in transconjugant strains. Undigested PFGE samples (a) and XhoI-digested samples (b) were transferred to nylon membranes and hybridized to *bont/a4* probes. pCLJ, at ~270 kb, is marked with an arrow. P, *C. botulinum* 657Ba (pCLJ-Erm) parent strain; D, pCLJ auxotrophic donor strain; R, recipient strains; T, transconjugant strains.



Supplementary Figure S4. Transconjugant strains are converted to toxigenicity. Samples of donor strain EMN053, recipient strain *C. sporogenes* PA3679, and transconjugant strain *C. sporogenes* PA3679 (pCLJ) were collected at 48 h and prepared as described in the text. S, supernatant; P, pellet; T, trypsinized supernatant. Gels were (a) stained to visualize total proteins or (b) immunoblotted for the presence of BoNT/A. Purified BoNT/A4 was used as a control. Samples were reduced with 100 mM DTT as indicated.



Supplementary Figure S5. Transconjugant strains can be identified by immunoblot. Representative donor, recipient, and transconjugant strains were grown for 96 h in TPGY and sampled and analyzed as described in the methods. All culture lysates were reduced with 100 mM DTT; the BoNT/A toxin controls were divided and only one portion, at right, was reduced. WT, *C. botulinum* 657Ba wild-type; P, *C. botulinum* 657Ba (pCLJ-Erm) parent strain; D, auxotrophic donor; R, recipient; T, transconjugant; M, Magic Mark XP Western Protein Standard.

Works Cited

1. Rossetto O, Megighian A, Scorzeto M, Montecucco C. Botulinum neurotoxins. Toxicon. 2013;67:31-6. Epub 2013/02/26. doi: 10.1016/j.toxicon.2013.01.017. PubMed PMID: 23435262.
2. Keesey J. The most vulnerable synapse: historic aspects of neuromuscular junction disorders. Handbook of clinical neurology. 2008;91:1-25. Epub 2008/07/18. doi: 10.1016/S0072-9752(07)01501-1. PubMed PMID: 18631839.
3. Rosow LK, Strober JB. Infant botulism: review and clinical update. Pediatr Neurol. 2015;52(5):487-92. Epub 2015/04/18. doi: 10.1016/j.pediatrneurol.2015.01.006. PubMed PMID: 25882077.
4. Smith TJ, Hill KK, Raphael BH. Historical and current perspectives on *Clostridium botulinum* diversity. Res Microbiol. 2015;166(4):290-302. Epub 2014/10/15. doi: 10.1016/j.resmic.2014.09.007. PubMed PMID: 25312020.
5. Johnson EA, Bradshaw M. *Clostridium botulinum* and its neurotoxins: a metabolic and cellular perspective. Toxicon. 2001;39(11):1703-22. Epub 2001/10/12. doi: 10.1016/S0041-0101(01)00157-X. PubMed PMID: 11595633.
6. Connan C, Popoff MR. Two-component systems and toxinogenesis regulation in *Clostridium botulinum*. Res Microbiol. 2015;166(4):332-43. Epub 2015/01/17. doi: 10.1016/j.resmic.2014.12.012. PubMed PMID: 25592073.
7. Mansfield MJ, Adams JB, Doxey AC. Botulinum neurotoxin homologs in non-*Clostridium* species. FEBS Lett. 2015;589(3):342-8. Epub 2014/12/30. doi: 10.1016/j.febslet.2014.12.018. PubMed PMID: 25541486.
8. Zornetta I, Azarnia Tehran D, Arrigoni G, Anniballi F, Bano L, Leka O, Zanotti G, Binz T, Montecucco C. The first non Clostridial botulinum-like toxin cleaves VAMP within the juxtamembrane domain. Scientific reports. 2016;6:30257. Epub 2016/07/23. doi: 10.1038/srep30257. PubMed PMID: 27443638; PMCID: PMC4957215.
9. Zhang S, Masuyer G, Zhang J, Shen Y, Lundin D, Henriksson L, Miyashita SI, Martinez-Carranza M, Dong M, Stenmark P. Identification and characterization of a novel botulinum neurotoxin. Nature communications. 2017;8:14130. Epub 2017/08/05. doi: 10.1038/ncomms14130. PubMed PMID: 28770820; PMCID: PMC5543303.
10. Carter AT, Paul CJ, Mason DR, Twine SM, Alston MJ, Logan SM, Austin JW, Peck MW. Independent evolution of neurotoxin and flagellar genetic loci in proteolytic *Clostridium botulinum*. BMC Genomics. 2009;10:115. Epub 2009/03/21. doi: 10.1186/1471-2164-10-115. PubMed PMID: 19298644; PMCID: PMC2674064.
11. Carter AT, Peck MW. Genomes, neurotoxins and biology of *Clostridium botulinum* Group I and Group II. Res Microbiol. 2015;166(4):303-17. Epub 2014/12/03. doi: 10.1016/j.resmic.2014.10.010. PubMed PMID: 25445012; PMCID: PMC4430135.
12. Weigand MR, Pena-Gonzalez A, Shirey TB, Broeker RG, Ishaq MK, Konstantinidis KT, Raphael BH. Implications of Genome-Based Discrimination between *Clostridium botulinum* Group I and *Clostridium sporogenes* Strains for Bacterial Taxonomy. Appl Environ Microbiol. 2015;81(16):5420-9. Epub 2015/06/07. doi: 10.1128/AEM.01159-15. PubMed PMID: 26048939; PMCID: PMC4510194.
13. Hill KK, Xie G, Foley BT, Smith TJ, Munk AC, Bruce D, Smith LA, Brettin TS, Detter JC. Recombination and insertion events involving the botulinum neurotoxin complex genes in *Clostridium botulinum* types A, B, E and F and *Clostridium butyricum* type E strains. BMC Biol. 2009;7:66. Epub 2009/10/07. doi: 10.1186/1741-7007-7-66. PubMed PMID: 19804621; PMCID: PMC2764570.

14. Dineen SS, Bradshaw M, Johnson EA. Neurotoxin gene clusters in *Clostridium botulinum* type A strains: sequence comparison and evolutionary implications. Curr Microbiol. 2003;46(5):345-52. Epub 2003/05/07. doi: 10.1007/s00284-002-3851-1. PubMed PMID: 12732962.
15. Frankel G, Newton SM, Schoolnik GK, Stocker BA. Intragenic recombination in a flagellin gene: characterization of the H1-j gene of *Salmonella typhi*. EMBO J. 1989;8(10):3149-52. Epub 1989/10/01. PubMed PMID: 2583095; PMCID: PMC401396.
16. Harrington CS, Thomson-Carter FM, Carter PE. Evidence for recombination in the flagellin locus of *Campylobacter jejuni*: implications for the flagellin gene typing scheme. J Clin Microbiol. 1997;35(9):2386-92. Epub 1997/09/01. PubMed PMID: 9276421; PMCID: PMC229973.
17. Smith NH, Beltran P, Selander RK. Recombination of *Salmonella* phase 1 flagellin genes generates new serovars. J Bacteriol. 1990;172(5):2209-16. Epub 1990/05/01. PubMed PMID: 2129534; PMCID: PMC208845.
18. Wassenaar TM, Fry BN, van der Zeijst BA. Variation of the flagellin gene locus of *Campylobacter jejuni* by recombination and horizontal gene transfer. Microbiology. 1995;141 (Pt 1):95-101. Epub 1995/01/01. doi: 10.1099/00221287-141-1-95. PubMed PMID: 7894725.
19. Smith TJ, Hill KK, Foley BT, Detter JC, Munk AC, Bruce DC, Doggett NA, Smith LA, Marks JD, Xie G, Brettin TS. Analysis of the neurotoxin complex genes in *Clostridium botulinum* A1-A4 and B1 strains: BoNT/A3, /Ba4 and /B1 clusters are located within plasmids. PLoS One. 2007;2(12):e1271. Epub 2007/12/07. doi: 10.1371/journal.pone.0001271. PubMed PMID: 18060065; PMCID: PMC2092393.
20. Weickert MJ, Chambliss GH, Sugiyama H. Production of toxin by *Clostridium botulinum* type A strains cured by plasmids. Appl Environ Microbiol. 1986;51(1):52-6. Epub 1986/01/01. PubMed PMID: 3082278; PMCID: PMC238814.
21. Marshall KM, Bradshaw M, Pellett S, Johnson EA. Plasmid encoded neurotoxin genes in *Clostridium botulinum* serotype A subtypes. Biochem Biophys Res Commun. 2007;361(1):49-54. Epub 2007/07/31. doi: 10.1016/j.bbrc.2007.06.166. PubMed PMID: 17658467; PMCID: PMC2346372.
22. Wisniewski JA, Rood JI. The Tcp conjugation system of *Clostridium perfringens*. Plasmid. 2017;91:28-36. Epub 2017/03/14. doi: 10.1016/j.plasmid.2017.03.001. PubMed PMID: WOS:000403193700005.
23. Franciosa G, Maugliani A, Scalfaro C, Aureli P. Evidence that plasmid-borne botulinum neurotoxin type B genes are widespread among *Clostridium botulinum* serotype B strains. PLoS One. 2009;4(3):e4829. Epub 2009/03/17. doi: 10.1371/journal.pone.0004829. PubMed PMID: 19287483; PMCID: PMC2653641.
24. Hosomi K, Sakaguchi Y, Kohda T, Gotoh K, Motooka D, Nakamura S, Umeda K, Iida T, Kozaki S, Mukamoto M. Complete nucleotide sequence of a plasmid containing the botulinum neurotoxin gene in *Clostridium botulinum* type B strain 111 isolated from an infant patient in Japan. Mol Genet Genomics. 2014;289(6):1267-74. Epub 2014/08/26. doi: 10.1007/s00438-014-0887-4. PubMed PMID: 25149145.
25. Skarin H, Hafstrom T, Westerberg J, Segerman B. *Clostridium botulinum* group III: a group with dual identity shaped by plasmids, phages and mobile elements. BMC Genomics. 2011;12:185. Epub 2011/04/14. doi: 10.1186/1471-2164-12-185. PubMed PMID: 21486474; PMCID: PMC3098183.
26. Zhou Y, Sugiyama H, Nakano H, Johnson EA. The genes for the *Clostridium botulinum* type G toxin complex are on a plasmid. Infect Immun. 1995;63(5):2087-91. Epub 1995/05/01. PubMed PMID: 7729925; PMCID: PMC173270.

27. Espelund M, Klaveness D. Botulism outbreaks in natural environments - an update. Front Microbiol. 2014;5:287. Epub 2014/06/27. doi: 10.3389/fmicb.2014.00287. PubMed PMID: 24966853; PMCID: PMC4052663.
28. Metcalf JL, Xu ZZ, Weiss S, Lax S, Van Treuren W, Hyde ER, Song SJ, Amir A, Larsen P, Sangwan N, Haarmann D, Humphrey GC, Ackermann G, Thompson LR, Lauber C, Bibat A, Nicholas C, Gebert MJ, Petrosino JF, Reed SC, Gilbert JA, Lynne AM, Bucheli SR, Carter DO, Knight R. Microbial community assembly and metabolic function during mammalian corpse decomposition. Science. 2016;351(6269):158-62. Epub 2015/12/15. doi: 10.1126/science.aad2646. PubMed PMID: 26657285.
29. von Wintersdorff CJ, Penders J, van Niekerk JM, Mills ND, Majumder S, van Alphen LB, Savelkoul PH, Wolffs PF. Dissemination of Antimicrobial Resistance in Microbial Ecosystems through Horizontal Gene Transfer. Front Microbiol. 2016;7(173):173. Epub 2016/03/01. doi: 10.3389/fmicb.2016.00173. PubMed PMID: 26925045; PMCID: PMC4759269.
30. Montecucco C, Rasotto MB. On botulinum neurotoxin variability. MBio. 2015;6(1). Epub 2015/01/08. doi: 10.1128/mBio.02131-14. PubMed PMID: 25564463; PMCID: PMC4313909.
31. Collins MD, East AK. Phylogeny and taxonomy of the food-borne pathogen *Clostridium botulinum* and its neurotoxins. J Appl Microbiol. 1998;84(1):5-17. Epub 2004/07/13. doi: 10.1046/j.1365-2672.1997.00313.x. PubMed PMID: 15244052.
32. Marshall KM, Bradshaw M, Johnson EA. Conjugative botulinum neurotoxin-encoding plasmids in *Clostridium botulinum*. PLoS One. 2010;5(6):e11087. Epub 2010/06/17. doi: 10.1371/journal.pone.0011087. PubMed PMID: 20552020; PMCID: PMC2884020.
33. Lin WJ, Johnson EA. Transposon Tn*916* mutagenesis in *Clostridium botulinum*. Appl Environ Microbiol. 1991;57(10):2946-50. Epub 1991/10/01. PubMed PMID: 1660696; PMCID: PMC183902.
34. Naglich JG, Andrews RE. Tn*916*-Dependent Conjugal Transfer of pC194 and pUB110 from *Bacillus subtilis* into *Bacillus thuringiensis* subsp. *israelensis*. Plasmid. 1988;20(2):113-26. Epub 1988/09/01. doi: Doi 10.1016/0147-619x(88)90014-5. PubMed PMID: WOS:A1988R376100003.
35. Showsh SA, Andrews RE, Jr. Analysis of the requirement for a pUB110 mob region during Tn*916*-dependent mobilization. Plasmid. 1999;41(3):179-86. Epub 1999/06/15. doi: 10.1006/plas.1999.1398. PubMed PMID: 10366523.
36. Bradshaw M, Tepp WH, Whitemarsh RC, Pellett S, Johnson EA. Holotoxin Activity of Botulinum Neurotoxin Subtype A4 Originating from a Nontoxigenic *Clostridium botulinum* Expression System. Appl Environ Microbiol. 2014;80(23):7415-22. Epub 2014/09/23. doi: 10.1128/AEM.01795-14. PubMed PMID: 25239905; PMCID: PMC4249187.
37. Whitemarsh RC, Tepp WH, Johnson EA, Pellett S. Persistence of botulinum neurotoxin A subtypes 1-5 in primary rat spinal cord cells. PLoS One. 2014;9(2):e90252. Epub 2014/03/04. doi: 10.1371/journal.pone.0090252. PubMed PMID: 24587301; PMCID: PMC3937374.
38. Whitmer ME, Johnson EA. Development of Improved Defined Media for *Clostridium botulinum* Serotype-A, Serotype-B, and Serotype-E. Applied and Environmental Microbiology. 1988;54(3):753-9. Epub 1988/03/01. PubMed PMID: WOS:A1988M313600022; PMCID: PMC202536.
39. Heap JT, Pennington OJ, Cartman ST, Carter GP, Minton NP. The ClosTron: a universal gene knock-out system for the genus *Clostridium*. J Microbiol Methods. 2007;70(3):452-64. Epub 2007/07/31. doi: 10.1016/j.mimet.2007.05.021. PubMed PMID: 17658189.
40. Foster PL. In vivo mutagenesis. Methods Enzymol. 1991;204:114-25. Epub 1991/01/01. PubMed PMID: 1943775; PMCID: PMC2994264.

41. Artış Ü. Enhanced butanol production by mutant strains of *Clostridium acetobutylicum* in molasses medium. Türk Biyokimya Dergisi (Turkish Journal of Biochemistry–Turk J Biochem). 2008;33(1):25-30.
42. Johnson EA, Tepp WH, Bradshaw M, Gilbert RJ, Cook PE, McIntosh ED. Characterization of *Clostridium botulinum* strains associated with an infant botulism case in the United Kingdom. J Clin Microbiol. 2005;43(6):2602-7. Epub 2005/06/16. doi: 10.1128/JCM.43.6.2602-2607.2005. PubMed PMID: 15956371; PMCID: PMC1151885.
43. Chomczynski P. One-hour downward alkaline capillary transfer for blotting of DNA and RNA. Anal Biochem. 1992;201(1):134-9. Epub 1992/02/14. PubMed PMID: 1621951.
44. Schantz EJ, Kautter DA. Standardized assay for *Clostridium botulinum* toxins [in foods].1978;v. 61.
45. Hatheway CL. Botulism. In: Balows A, Hausler WJ, Ohashi M, Turano A, Lennete EH, editors. Laboratory Diagnosis of Infectious Diseases. New York, NY: Springer New York; 1988. p. 111-33.
46. Maslanka SE, Solomon HM, Sharma S, Johnson EA. *Clostridium botulinum* and its toxins. Compendium of methods for the microbiological examination of foods American Public Health Association, Washington, DC. 2013.
47. Schantz EJ, Johnson EA. Properties and Use of Botulinum Toxin and Other Microbial Neurotoxins in Medicine. Microbiological Reviews. 1992;56(1):80-99. PubMed PMID: WOS:A1992HH37500005.
48. Binz T, Kurazono H, Wille M, Frevert J, Wernars K, Niemann H. The complete sequence of botulinum neurotoxin type A and comparison with other clostridial neurotoxins. J Biol Chem. 1990;265(16):9153-8. Epub 1990/06/05. PubMed PMID: 2160960.
49. Johnson EA, Lin WJ, Zhou YT, Bradshaw M. Characterization of neurotoxin mutants in *Clostridium botulinum* type A. Clin Infect Dis. 1997;25 Suppl 2:S168-70. Epub 1997/10/06. PubMed PMID: 9310666.
50. Williamson CHD, Sahl JW, Smith TJ, Xie G, Foley BT, Smith LA, Fernandez RA, Lindstrom M, Korkeala H, Keim P, Foster J, Hill K. Comparative genomic analyses reveal broad diversity in botulinum-toxin-producing Clostridia. Bmc Genomics. 2016;17(1):180. Epub 2016/03/05. doi: ARTN 180 10.1186/s12864-016-2502-z. PubMed PMID: WOS:000371595000006; PMCID: PMC4778365.
51. Schill KM, Wang Y, Butler RR, 3rd, Pombert JF, Reddy NR, Skinner GE, Larkin JW. Genetic Diversity of *Clostridium sporogenes* PA 3679 Isolates Obtained from Different Sources as Resolved by Pulsed-Field Gel Electrophoresis and High-Throughput Sequencing. Appl Environ Microbiol. 2016;82(1):384-93. Epub 2015/11/01. doi: 10.1128/AEM.02616-15. PubMed PMID: 26519392; PMCID: PMC4702626.
52. Wang X, Maegawa T, Karasawa T, Kozaki S, Tsukamoto K, Gyobu Y, Yamakawa K, Oguma K, Sakaguchi Y, Nakamura S. Genetic analysis of type E botulinum toxin-producing *Clostridium butyricum* strains. Appl Environ Microbiol. 2000;66(11):4992-7. Epub 2000/10/31. doi: 10.1128/aem.66.11.4992-4997.2000. PubMed PMID: 11055954; PMCID: PMC92410.
53. Giordani F, Fillo S, Anselmo A, Palozzi AM, Fortunato A, Gentile B, Azarnia Tehran D, Ciammaruconi A, Spagnolo F, Pittiglio V, Anniballi F, Auricchio B, De Medici D, Lista F. Genomic characterization of Italian *Clostridium botulinum* group I strains. Infect Genet Evol. 2015;36:62-71. Epub 2015/09/06. doi: 10.1016/j.meegid.2015.08.042. PubMed PMID: 26341861.
54. Hunter SB, Vauterin P, Lambert-Fair MA, Van Duyne MS, Kubota K, Graves L, Wrigley D, Barrett T, Ribot E. Establishment of a universal size standard strain for use with the PulseNet standardized pulsed-field gel electrophoresis protocols: converting the national databases to the

new size standard. J Clin Microbiol. 2005;43(3):1045-50. Epub 2005/03/08. doi: 10.1128/JCM.43.3.1045-1050.2005. PubMed PMID: 15750058; PMCID: PMC1081233.
55. Skarin H, Segerman B. Plasmidome interchange between *Clostridium botulinum*, *Clostridium novyi* and *Clostridium haemolyticum* converts strains of independent lineages into distinctly different pathogens. PLoS One. 2014;9(9):e107777. Epub 2014/09/26. doi: 10.1371/journal.pone.0107777. PubMed PMID: 25254374; PMCID: PMC4177856.
56. Ng V, Lin WJ. Comparison of assembled *Clostridium botulinum* A1 genomes revealed their evolutionary relationship. Genomics. 2014;103(1):94-106. Epub 2013/12/27. doi: 10.1016/j.ygeno.2013.12.003. PubMed PMID: 24369123; PMCID: PMC3959226.
57. Dover N, Barash JR, Hill KK, Xie G, Arnon SS. Molecular characterization of a novel botulinum neurotoxin type H gene. J Infect Dis. 2014;209(2):192-202. Epub 2013/10/10. doi: 10.1093/infdis/jit450. PubMed PMID: 24106295.
58. Grohmann E, Muth G, Espinosa M. Conjugative plasmid transfer in gram-positive bacteria. Microbiol Mol Biol Rev. 2003;67(2):277-301, table of contents. Epub 2003/06/10. doi: 10.1128/mmbr.67.2.277-301.2003. PubMed PMID: 12794193; PMCID: PMC156469.
59. Li J, Adams V, Bannam TL, Miyamoto K, Garcia JP, Uzal FA, Rood JI, McClane BA. Toxin plasmids of *Clostridium perfringens*. Microbiol Mol Biol Rev. 2013;77(2):208-33. Epub 2013/05/24. doi: 10.1128/MMBR.00062-12. PubMed PMID: 23699255; PMCID: PMC3668675.
60. Wisniewski JA, Traore DA, Bannam TL, Lyras D, Whisstock JC, Rood JI. TcpM: a novel relaxase that mediates transfer of large conjugative plasmids from *Clostridium perfringens*. Molecular Microbiology. 2016;99(5):884-96. Epub 2015/11/13. doi: 10.1111/mmi.13270. PubMed PMID: WOS:000371241600007.
61. Adams V, Li J, Wisniewski JA, Uzal FA, Moore RJ, McClane BA, Rood JI. Virulence Plasmids of Spore-Forming Bacteria. Microbiol Spectr. 2014;2(6). Epub 2015/06/25. doi: 10.1128/microbiolspec.PLAS-0024-2014. PubMed PMID: 26104459.
62. Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. Basic local alignment search tool. J Mol Biol. 1990;215(3):403-10. Epub 1990/10/05. doi: 10.1016/S0022-2836(05)80360-2. PubMed PMID: 2231712.
63. Lauber CL, Metcalf JL, Keepers K, Ackermann G, Carter DO, Knight R. Vertebrate decomposition is accelerated by soil microbes. Appl Environ Microbiol. 2014;80(16):4920-9. Epub 2014/06/08. doi: 10.1128/AEM.00957-14. PubMed PMID: 24907317; PMCID: PMC4135757.
64. Metcalf JL, Carter DO, Knight R. Microbiology of death. Current biology : CB. 2016;26(13):R561-R3. Epub 2016/07/13. doi: 10.1016/j.cub.2016.03.042. PubMed PMID: 27404249.
65. Smith L, Sugiyama H. The natural occurrence of *Clostridium botulinum*. Botulism: The Organism, its Toxins, the Disease. 1988.

Chapter 2: Molecular mechanism of plasmid transfer in *Clostridium botulinum*

Nawrocki EM, Fredrick CM, Bradshaw M, Johnson EA

Manuscript in preparation

Author Contributions

E.M.N. designed and conducted experiments and drafted the manuscript with input from all authors. C.M.F. and M.B. generated the Tm^R ClosTron marker and constructed various *C. botulinum* mutant strains. E.A.J. assisted in the supervision and critique of the research.

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Additional Information

The authors declare that they have no conflicts of interest.

Abstract

Botulinum neurotoxins (BoNTs), once thought to be exclusive to the species *Clostridium botulinum*, are among the world's most potent toxins. The discovery of novel BoNTs, the presence of botulinum-like toxins outside *C. botulinum*, and the genomic diversity of the species itself all suggest that the *bont* gene may be subject to horizontal gene transfer. Prior studies demonstrated that BoNT-encoding plasmids could be mobilized from their Group I *C. botulinum* host strains to various clostridial recipients. Here, we identify a gene on one such plasmid, pCLJ, that is necessary for the plasmid's transfer. Using the original ClosTron method of insertional inactivation to mutagenize plasmid genes and a derivative of the ClosTron to tag a representative recipient strain with a second selectable marker, we show that the inactivation of CLJ_0213 is not permissive to conjugation. The conjugation defect can be partially rescued by complementation with a copy of CLJ_0213 in *trans*. CLJ_0213, a putative ATPase, is the first gene outside the toxin complex on pCLJ to be functionally characterized. The identification of a gene essential for pCLJ conjugation aids in defining the molecular mechanism of plasmid transfer in *C. botulinum*. As the presence of CLJ_0213 homologues on other plasmids with *bont* genes could be indicative of their propensity to transfer between strains, this finding may prove useful to the surveillance and detection of neurotoxigenic clostridia.

Introduction

Clostridium botulinum is an anaerobic Gram-positive bacterium most notable for producing botulinum neurotoxin (BoNT), the causative agent of botulism. The organism and its toxins are genetically and physiologically diverse: *C. botulinum* is the species designation given to four distinct groups of bacteria (Groups I-IV), and BoNTs exist in at least seven serotypes (BoNT/A through BoNT/G) (1). *C. botulinum* spores are common in dust, soil, and sediment (2-4). In rare cases, they may germinate and grow in the gastrointestinal tracts of mammals or in deep wounds, upon which BoNTs may enter the bloodstream, travel to neurons, and cleave SNARE proteins, paralyzing the animal (5). Botulism can also be caused by ingestion of pre-formed BoNT, which may be present in improperly prepared foods (6). Though cases of human botulism are infrequent, they require intensive treatment without which they are often lethal (6, 7). *C. botulinum* and purified BoNTs are therefore subject to strict scrutiny under the Centers for Disease Control and Prevention's Select Agent Program and are classified as Tier 1 Select Agents.

Of the four physiological Groups of *C. botulinum*, Group I is predominantly responsible for human botulism (3, 8). These bacteria grow optimally at 37°C and are closely related to *C. sporogenes* (9). Group I *C. botulinum* strains may produce BoNT/A, BoNT/B, or BoNT/F. Some strains are multivalent, including *C. botulinum* 657Ba, which encodes both BoNT/A and BoNT/B on a large extrachromosomal element (10). Previous studies in our laboratory identified the presence of toxin genes on this plasmid (pCLJ) and demonstrated its transfer to a number of other clostridial strains (11-13). Strains in all four Groups of *C. botulinum* are now known to possess BoNT-encoding plasmids or bacteriophage (1, 14, 15). Strains outside of *C. botulinum*, including isolates of *C. butyricum*, *C. baratii*, and *C. sporogenes*, are also capable of maintaining

and producing the neurotoxin (16). The acquisition of novel BoNTs via mobile genetic elements may contribute to the diversity of these toxins across the clostridia (17).

Recent genome sequences have also revealed the presence of botulinum-like toxins in non-clostridial organisms (18-21). The activity and specificity of these putative new toxins are topics of keen interest to the botulism research community. In one case, a botulinum-like toxin sequence is found on an *Enterococcus faecium* plasmid that appears to be conjugative (20). The transfer of this plasmid or others encoding BoNTs to bacterial strains that are more infective, invasive, or antibiotic-resistant than their native hosts would pose an alarming threat to human and animal health and safety.

The mobility of BoNT-encoding plasmids is both suggested by genomics and supported experimentally, but the mechanism by which they are transferred has been poorly characterized. At minimum, a mobilizable plasmid will contain an origin of transfer and a relaxase protein that cleaves DNA within the *oriT*; it may also contain a type IV coupling protein that connects relaxosome components (i.e. DNA and bound protein) to the membrane-bound mating channel (22). A conjugative or self-transmissible plasmid encodes the proteins of the mating channel complex as well, which are classified as a type IV secretion system (22). In clostridia, conjugative plasmids are typified by pCW3 of *C. perfringens*, which within the *tcp* locus encodes a relaxase (TcpM), a coupling protein (TcpA), and multiple core components of the mating channel (23).

The sequences of pCLJ and other *C. botulinum* plasmids are replete with annotations of hypothetical proteins, including some with domain homology to secretion system components, but no canonical conjugation apparatus has been identified (10, 24). Having observed the mobility of pCLJ, we set out to investigate the genes responsible for its transfer. Utilizing two ClosTron markers, one of which was modified in-house, we here show that CLJ_0213, a putative

ATPase in the “conjugation region” of pCLJ, is necessary for said plasmid’s transfer to a representative *C. botulinum* recipient.

Methods

Biosafety and biosecurity

Clostridium botulinum and BoNTs are classified as Tier 1 Select Agents, the highest security group of biological agents. The Johnson laboratory and personnel are registered with the Federal Select Agent Program for research involving BoNTs and BoNT-producing strains of clostridia. The research program, procedures, documentation, security, and facilities are closely monitored by the University of Wisconsin-Madison Biosecurity Task Force, University of Wisconsin-Madison Office of Biological Safety, University of Wisconsin Select Agent Program, and the Centers for Disease Control and Prevention. All personnel continually undergo suitability assessments and rigorous and biosafety training, including biosafety level 3 (BSL3) or BSL2 and select agent practices, before participating in laboratory studies involving BoNTs and neurotoxigenic *C. botulinum*.

Bacterial strains and culture conditions

Clostridial strains were maintained at 37°C on the following media: liquid cultures in TPGY (50 g/l trypticase peptone, 5 g/l Bacto peptone, 4 g/l dextrose, 20 g/l yeast extract, 1 g/l cysteine-HCl, pH 7.4) and solid cultures on TYG (30 g/l Bacto tryptone, 20 g/l yeast extract, 1 g/l sodium thioglycolate) prepared with 1.5% agar. Cloning hosts (e.g. *E. coli* 10-beta, New England Biolabs, Ipswich, MA) were maintained at 37°C in standard rich media (LB or 2×YT). Frozen stocks were supplemented with 20% glycerol and stored at -80°C. Antibiotics were used at the following concentrations: erythromycin at 50 μg/ml, chloramphenicol at 25 μg/ml in solid culture and 12.5 μg/ml in liquid culture, thiamphenicol at 15 μg/ml, spectinomycin at 250 μg/ml for *E. coli* and 600 μg/ml for *C. botulinum*. In mixed matings between *E. coli* and clostridia, the latter were selected with the addition of 250 μg/ml cycloserine. All chemicals and media

components were purchased from Becton Dickinson Microbiology Systems (Sparks, MD) and Sigma-Aldrich (St. Louis, MO) unless otherwise noted.

Clostridial cultures were grown under anaerobic conditions. Glass culture tubes were flushed with nitrogen gas and sealed with butyl rubber stoppers (Bellco Glass, Vineland, NJ) before sterilizing. All culture manipulations were performed in an anaerobic chamber (Forma Anaerobic System, Marietta, OH) with an initial gas mixture of 80% N_2, 10% CO_2, and 10% H_2. Resazurin was added to solid media at 2 μg/ml, and agar plates were prereduced by overnight incubation in the anaerobic chamber.

ClosTron mutagenesis and modification

pCLJ genes with possible roles in conjugation were identified based on plasmid synteny in Group I *C. botulinum*. In prior mating experiments, both pCLJ (from strain 657Ba) and pCLK (from strain CDC-A3) were mobilized to *C. botulinum* recipient strains (12). pCLD, from *C. botulinum* strain Okra, was not. pCLD is missing a 70 kb fragment that is now referred to as the conjugation region, as it encodes many ORFs with similarity to secretion system components (24). Genes in the conjugation region that are conserved on pCLJ and pCLK were chosen for mutagenesis and inactivated via ClosTron insertion as previously described (25). Briefly, targeting constructs were designed algorithmically (see clostron.com) and purchased from DNA2.0 (Table 1). The retargeted ClosTron vectors were transformed into *E. coli* CA434 and transferred by conjugation to the desired *C. botulinum* strain (26). Transformants were recovered on media selective for the vector, then replica plated on media selective for the retrotransposition-activated marker (RAM; Table 1). Once the desired intron insertion was verified by PCR (Table 2), isolates were restreaked to screen for loss of the targeting vector.

Toxin plasmids were tagged with derivatives of the pMTL007C-E2 ClosTron, conferring erythromycin resistance. In order to identify transconjugants that obtained such plasmids, a second selective marker was constructed by swapping the ClosTron's *ermB* and *catP* genes and was used to inactivate the *bont/a* gene on the chromosome of Hall A-*hyper*. This thiamphenicol-resistant strain served as a representative *C. botulinum* plasmid recipient. A short description of the cloning strategy follows.

First, an MluI digest of the ClosTron vector pMTL007C-E2:botA580s (DNA2.0) (27) was performed to excise the *ermB*-RAM. The plasmid backbone was then re-ligated to generate a RAM-less vector, pMTL007C-ΔRAM. Next, the antibiotic resistance module containing the *ermB* gene was excised from the pMTL80000 modular vector series via PmeI-FseI digest and ligated into the same restriction sites on pMTL007C-ΔRAM, replacing the *catP* gene on the vector backbone and creating pMTL007E-ΔRAM. Finally, a new *catP* RAM including 12 extraneous 5' codons identical to those of the *ermB*-RAM was generated by PCR (25); Table 2. Three overlapping PCRs were used to construct a fragment containing the *catP* RAM and its requisite upstream and downstream flanking regions extending to the MluI sites. The resulting PCR product and the pMTL007E-ΔRAM backbone were digested with MluI and ligated to create pMTL007E-C2:botA580s, an inversion of the original ClosTron targeting vector.

Table 1. Plasmid vectors. pMTL007C-E2 plasmids were purchased from DNA2.0 and used to generate mutations in the conjugation region of pCLJ. pMTL007E-C2 was modified as described in the text and used to generate a suitable recipient strain for conjugal matings.

Name	Description	Source
pMTL007C-E2:botA580s	ClosTron plasmid (*ermB* RAM, *catP* vector) targeting the *botA* gene of *C. botulinum* Hall A-*hyper*	(25); (27)
pMTL007C-E2:CLJ_0213-394s	ClosTron plasmid (*ermB* RAM, *catP* vector) targeting the CLJ_0213 gene of *C. botulinum* 657Ba	This work
pMTL007C-E2:CLJ_0227-229s	ClosTron plasmid (*ermB* RAM, *catP* vector) targeting the CLJ_0227 gene of *C. botulinum* 657Ba	This work
pMTL007C-E2:CLJ_0228-229s	ClosTron plasmid (*ermB* RAM, *catP* vector) targeting the CLJ_0228 gene of *C. botulinum* 657Ba	This work
pMTL007C-E2:CLJ_0231-387a	ClosTron plasmid (*ermB* RAM, *catP* vector) targeting the CLJ_0231 gene of *C. botulinum* 657Ba	This work
pMTL007E-C2:botA-580s	ClosTron plasmid (*catP* RAM, *ermB* vector) targeting the *botA* gene of *C. botulinum* Hall A-*hyper*	This work
pJET1.2-P2	*E. coli* cloning vector, Ap^R. Derived from pJET1.2 (ThermoFisher Scientific) by inserting the P_{thl} + MCS module from the pMTL80000 series at the blunt-end cloning site	(28)
pMTL83353	*E. coli-Clostridium* shuttle vector, Sp^R	(28)

Plasmid transfer

Donor and recipient strains were revived from frozen stock and streaked for isolated colonies. Single colonies were inoculated in TPGY broth, subcultured once, and grown to OD_{600} ≈ 0.8. In order to enumerate donors and recipients, 100 µl of each culture was spotted individually on TYG agar. To assess plasmid transfer, equal volumes (100 µl) of donor and recipient were spotted together on TYG agar. All agar plates were allowed to incubate at 37°C for 18 h. Bacterial growth was then washed with 3 ml 1 × PBS, and the suspensions were serially diluted in 1 × PBS. Dilutions of donor and recipient controls were plated on TYG + Em and TYG + Tm, respectively, to determine CFU/ml. Aliquots of the donor and recipient suspensions (100 µl) were also plated on TYG supplemented with inhibitory antibiotics (thiamphenicol and erythromycin, respectively) to account for spontaneous mutation. Mating mixtures were diluted and plated on TYG + Em + Tm to enumerate transconjugants. Transfer frequency was calculated by dividing transconjugant CFU/ml by the larger of the donor or recipient CFU/ml.

Vector construction and in silico analysis

Complementation vectors were constructed by amplifying the wild-type sequence of CLJ_0213 with primers designed to incorporate in-frame NdeI and NheI restriction sites (Table 2). Fragments generated by PCR were digested with these enzymes and ligated into the NdeI-NheI sites of pJET1.2-P2 (Table 1). The P2-ORF constructs were verified by Sanger sequencing (see primers in Table 2), then digested with SbfI and AscI and ligated into the modular clostridial expression vector pMTL83353 (28). The resulting construct is of the form pMTL83352:ORF, in which the native gene from start to stop codon is placed under the control of the high-activity thiolase promoter from *C. acetobutylicum* ATCC 824 (28, 29). This vector was introduced into *C. botulinum* mutants by conjugation from *E. coli* CA434 as previously described.

Transmembrane helices in the structure of CLJ_0213 were predicted using the TMHMM server v. 2.0 (http://www.cbs.dtu.dk/services/TMHMM/) (30). Truncations of CLJ_0213 were designed to delete the transmembrane helices and either the N-terminal (ΔTM1, ΔTM1TM2) or the C-terminal (ΔTM3) regions adjacent. These constructs were prepared using the ΔTM primers given in Table 2 and verified by Sanger sequencing.

Fitting its annotation as an ATPase, the sequence of CLJ_0213 contains Walker A and B motifs (GenBank ACQ51375.1). The annotated Walker A motif ($G_{172}T_{173}G_{174}K_{175}S_{176}$) was selected for mutagenesis and was validated as an ATP binding pocket in an online analysis by ATPbind (https://zhanglab.ccmb.med.umich.edu/ATPbind/) (31). A site-directed mutant of CLJ_0213, K175A, was generated by QuikChange mutagenesis (Agilent Biotechnologies, Santa Clara, CA). pJET1.2-CLJ_0213 served as template DNA and was mutagenized with the CLJ_0213-K175A-QC complementary primers according to the manufacturer's recommended protocol. The mutation was verified using the CLJ_0213-K175A-seq primer pair (Table 2).

Table 2. Oligonucleotide primers. Restriction sites are given in bold type. Overlapping sequences are underlined. Reaction conditions are available upon request. All oligonucleotides were purchased from Integrated DNA Technologies (Coralville, IA).

Name	Sequence
catP-overlapA-F	GTCGACATTCACTTGTGTTTATGAATCACGTGACG
catP-overlapA-R	CAATTTTTTCAAATACCATACCAGCACCAGAAGCACC
catP-overlapB-F	GGTGCTTCTGGTGCTGGTATGGTATTTGAAAAAATTGAT
catP-overlapB-R	GGAACTTCGCGACTCATAGAATTAACTATTTATCAATTC CTGC
catP-overlapC-F	GCAGGAATTGATAAATAGTTAATTCTATGAGTCGCGAAG TTCCTATTCTC
catP-overlapC-R	CAACAGTTGCGCAGCCTGAATG
BoNT/A-500F	GCTTTGGACATGAAGTTTTGAATC
BoNT/A-926R	GAAGCAGTAGTACCTACTATTGATTTA
CLJ_BvB-118F	ACAGATCGTATTTGGATAATACCGG
CLJ_BvB-677R	AAGGCTGGATCTGAAAAATATCCAC
CLJ_0213-90F	TGCTACTACCCCAGCAATTTTAGTA
CLJ_0213-518R	GTACCAGGAGCACCAAATATAGCTA
CLJ_0227-94F	CTTCCTCCATATCTTGTTGT
CLJ_0227-352R	GGTCTCCAAATAGGTATGCCTGTAT
CLJ_0228-199F	CGTAAATTTCTCAAAGCAGGTATAAACG
CLJ_0228-511R	ACTCTCCTTTTAATGGATAGGCTACA
CLJ_0231-173F	CTTATGCAGCTTCAAACAGCACTAT
CLJ_0231-497R	TAAGGCTCAATTGATTCAGGAGGAA
CLJ_0213-NdeI	**CATATG**AATATATTAAATATTTTAAAAAAGGC
CLJ_0213-NheI	**GCTAGC**TTAATTGAAAAAGTCATTTAGTG
CLJ_0213-K175A-QC	TACTGTTCTTAAATAGAATCTAGATGCACCAGTACCAGG AGCACCAAATAT
CLJ_0213-K175A-QC	ATATTTGGTGCTCCTGGTACTGGTGCATCTAGATTCTATT TAAGAACAGTA
CLJ_0213-K175A-seqF	ACGGAACACATGGAACTGCT
CLJ_0213-K175A-seqR	TCACCCTTTGAACCAACAGGT
CLJ_0213-DE421/422AA-seqF	TGCGCTACCTGGTGATCATC
CLJ_0213-DE421/422AA-seqR	TAATCCTTCTAAGCCGCCGC
CLJ_0213-ΔTM1-NdeI	**CATATG**AAAAATATGCTAGACCCTAAGAATAAAGG
CLJ_0213-ΔTM1TM2-NdeI	**CATATG**AATAAAGAAAAACAAGGAATAAATAACAATG
CLJ_0213-ΔTM3-NheI	**GCTAGC**TTAATCAAATGTTGAAGTCATATC
CLJ_0213-M28-NdeI	**CATATG**ATATATGCTACTACCCCAGC

Results

The catP-RAM is functional in C. botulinum

In order to assess the role of various genes in the pCLJ conjugation region, we designed a conjugation assay based on dual antibiotic selections. As before, pCLJ was tagged at desired loci with the *ermB* RAM of the Em^R ClosTron system (Table 1). The ClosTron was then reengineered in order to mark the chromosome of a recipient *C. botulinum* strain with thiamphenicol resistance. The RAM of the pMTL007C-E2 ClosTron vector carries a self-splicing *td* intron situated within a twelve-codon leader sequence preceding the *ermB* gene (25). When the *td* intron excises upon chromosomal integration, the resulting gene product is a modified Em^R protein with twelve extraneous amino acids at the N-terminus. When replacing the *ermB* gene with *catP*, the upstream leader sequence was preserved, and the *td* intron was linked to the gene in the same fashion. The modified *catP* gene was functional in *E. coli* and *C. botulinum*, suggesting that the addition of the twelve-codon leader sequence to the N-terminus was not deleterious to the Cm^R/Tm^R protein. The pMTL007E-C2 vector was subsequently used to inactivate the *bont/a* gene on the chromosome of *C. botulinum* Hall A-*hyper*. The insertion of the Tm^R ClosTron (approximately 1.8 kb) was confirmed by PCR and the lack of BoNT/A in the mutant was demonstrated by Western blot (Figure 1).

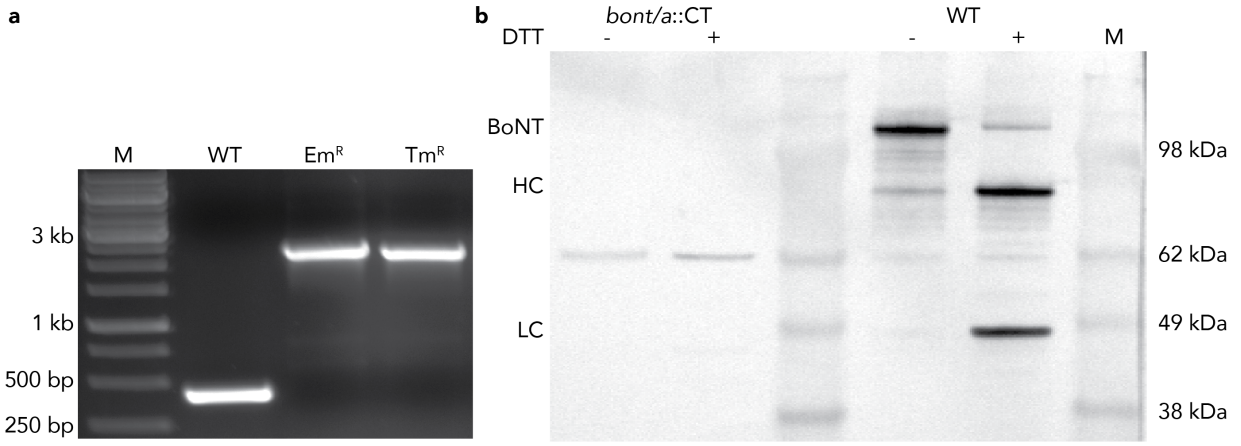

Figure 1. The ClosTron derivative pMTL007E-C2:botA580s is functional in *C. botulinum* Hall A-*hyper*. Construction of a Tm^R ClosTron marker enabled the generation of a nontoxigenic *C. botulinum* strain, to be used as a representative recipient in conjugal mating experiments. (a) The *ermB*-RAM (Em^R) and the *catP*-RAM (Tm^R) are each approximately 1.8 kb in size. Their insertion at the botA580s target site is demonstrated by PCR amplicons ~1.8 kb larger than wild-type (WT). M, 1 kb GeneRuler. (b) Insertional inactivation of the *bont/a* gene by the *catP*-RAM abolishes BoNT/A expression. No toxin-specific signals are detected via Western blot in the Tm^R ClosTron mutant (*bont/a*::CT). Samples were reduced with the addition of 100 mM DTT as indicated to cleave the disulfide bond linking the heavy chain (HC) and light chain (LC) of the BoNT. M, SeeBlue Plus2 Pre-stained Protein Standard.

CLJ_0213 is necessary for pCLJ transfer

The "conjugation region" of pCLJ, the large BoNT-encoding plasmid of *C. botulinum* strain 657Ba, contains several genes that bear a functional annotation pertaining to conjugation and have homology to other toxin plasmids in Group I *C. botulinum* (Table 1). Four of these genes were inactivated and tagged with an Em^R marker by ClosTron mutagenesis. Successful erythromycin-resistant ClosTron integrants were obtained in each case (Figure 2), indicating that none of the four pCLJ targets are essential genes under the given growth conditions. The conjugation region mutants were compared to *bont/b*::*ermB*, in which the major botulinum neurotoxin gene on the plasmid was inactivated via ClosTron. Inactivation of *bont/b* is permissive to pCLJ transfer (13). The nontoxigenic Tm^R *C. botulinum* Hall A-*hyper* strain (Figure 1) served as a representative recipient strain in solid agar matings. Transconjugants that

obtain the *ermB*-tagged plasmid from the donor strain are easily selected on double antibiotic (Em + Tm) media.

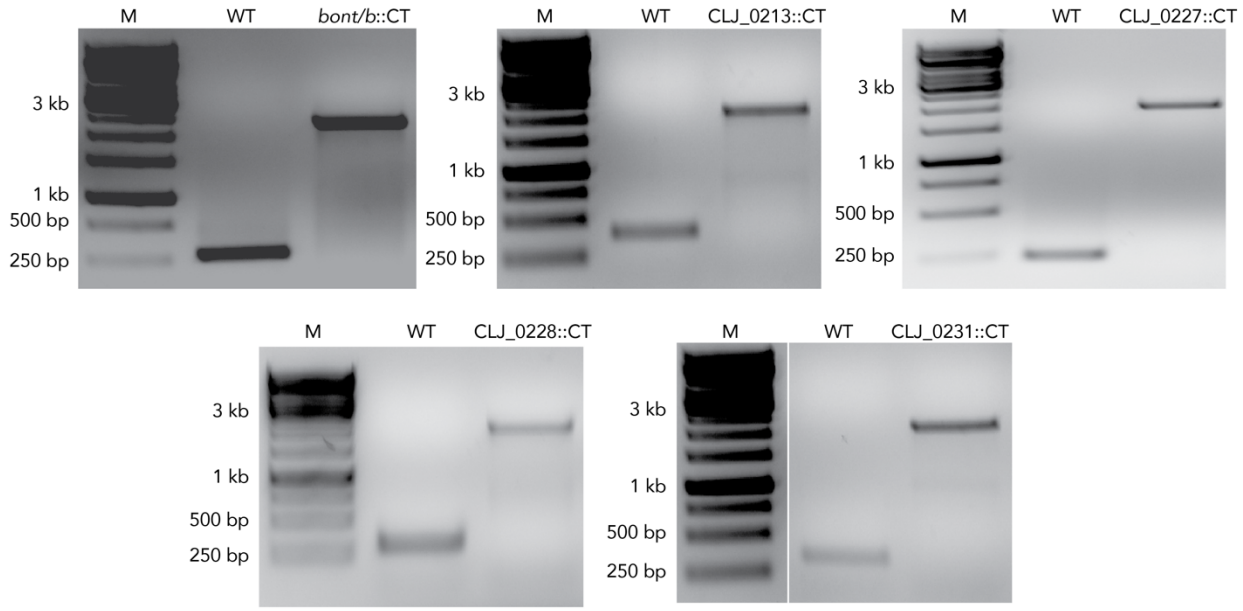

Figure 2. Putative conjugation genes on pCLJ are insertionally inactivated with the Em^R ClosTron. The given locus tags were targeted with a derivative of the pMTL007C-E2 ClosTron, carrying the *ermB*-RAM. PCRs of the target sites reveal 1.8 kb insertions relative to the wild-type (Table 2). Inactivation of *bont/b* was permissive to pCLJ transfer in a prior study (13). M, 1 kb GeneRuler.

Conjugation assays using the pCLJ mutants as donors yielded discrete, countable Em^{R}Tm^R colonies in nearly all instances (Table 3). While the transfer frequencies of pCLJ conjugation region mutants (defined as transconjugant CFU/ml divided by the larger of the donor or recipient CFU/ml) were lower than that of the baseline *bont/b*::*ermB* plasmid donor, Em^{R}Tm^R colonies were consistently recovered in quantities above the spontaneous mutation frequency, suggesting that the tagged plasmids were transferred to the recipient (Table 3). The sole exception was the CLJ_0213::*ermB* donor, which has generated no Em^{R}Tm^R colonies in any mating experiment performed to date (Table 3).

Table 3. pCLJ mutants transfer at various frequencies. Matings were performed on solid agar as described in the text. The given locus tags were inactivated by Em^R ClosTron mutagenesis. The Tm^R Cbo:*botA*-580s::CT mutant served as recipient in all mating pairs. Transfer frequency was determined by dividing transconjugant CFU/ml by the larger of the donor or recipient CFU/ml. Transfer frequencies reflect the means of independent assays, minimum $n = 2$.

Locus Tag (Gene Name)	Functional Annotation	Transfer Frequency
CLJ_0133 (*bont/b*)	Botulinum neurotoxin	5.5×10^{-8}
CLJ_0213	ATPase involved in conjugal plasmid transfer	TFTC*
CLJ_0227	Type II secretion system protein F domain-containing protein	4.9×10^{-11}
CLJ_0228	Type II secretion system protein F domain-containing protein	4.6×10^{-12}
CLJ_0231	Type II secretion system protein E domain-containing protein	1.4×10^{-12}

*TFTC, transconjugant colonies numbered too few to count

pCLJ transfer is restored by complementation

To assess whether the gene product of CLJ_0213 was necessary for conjugal transfer of pCLJ or alternatively, whether this phenotype was due to polar effects of the mutation, a complementation experiment was conducted providing the wild-type CLJ_0213 gene in *trans*. CLJ_0213::*ermB* was complemented by cloning the wild-type allele into the spectinomycin-resistant *E. coli-Clostridium* shuttle vector pMTL83352. The pMTL vector chosen for this application contains a pCB102 Gram-positive replicon, a ColE1 Gram-negative replicon, and an *oriT* and *traJ* from the RK2 plasmid (28). The presence of the RK2 elements implies that the plasmid can be conjugally transferred from an *E. coli* helper strain. Note that an Sp^R empty vector from this plasmid lineage does not permit the transfer of erythromycin resistance to a Tm^R recipient strain in clostridial matings (Table 4).

Complementation of CLJ_0213::*ermB* with the wild-type ORF (provided on pMTL83352 and expressed constitutively) does partially rescue the conjugation defect, giving Em^RTm^R transconjugant colonies in the conjugation assay (Table 4). Computational analysis of the CLJ_0213 ORF suggests that the resulting protein contains at least three transmembrane helices

(Figure 3a-b). Truncations that delete the transmembrane helices from the N-terminus of the protein do not rescue conjugation, as complementation with these constructs does not permit the development of Em^RTm^R transconjugant colonies (Figure 3a; Table 4). Similarly, a complementation construct with an alanine substitution in the active site of CLJ_0213 (K175A) does not allow the transfer of *ermB* to the Tm^R recipient (Figure 3a; Table 4).

The start codon of CLJ_0213 may be misannotated

While these results indicate a direct and essential role for CLJ_0213 in plasmid conjugation, surprisingly, resequencing of the full-length "wild-type" construct on the complementation vector used in these studies revealed that it had acquired a single mutation: one nucleotide was deleted from a stretch of seven adenines near the 5' end of the gene, a_{33}-a_{39}. The frameshift would result in a premature stop codon, closing the reading frame after the twenty-seventh amino acid (Figure 3c). As the adenine-deletion variant of the gene was still able to complement the CLJ_0213::*ermB* mutant, alternative reading frames were investigated. An obvious candidate is the third frame of the deletion variant, which initiates with a methionine (M28 of the WT) and terminates at the same stop codon as the wild-type (Figure 3c). To determine whether this translation is functional in *C. botulinum*, we designed a complementation construct that would mimic the frameshifted mutant: $CLJ\_0213_{28\text{-}648}$, which deletes the gene's first 27 codons and initiates with M28 (Figure 3c). The $CLJ\_0213_{28\text{-}648}$ construct also partially restored conjugation to the CLJ_0213::*ermB* mutant, although the observed transfer frequency was not as high as with the presumed full-length ORF (Table 4).

Table 4. Complementation can partially rescue the conjugation defect in CLJ_0213::*ermB*. Matings between the Em^R plasmid donor and the Tm^R recipient were performed as previously described. The CLJ_0213::*ermB* mutant was complemented in *trans* with the given constructs in the pMTL83352 Sp^R vector. The transfer frequency of pCLJ was determined by dividing the CFU/ml of Em^RTm^R transconjugants by the CFU/ml of Em^R donors. Transfer frequencies reflect the means of independent assays, minimum $n = 2$.

Donor Strain	Complementation Vector	Transfer Frequency
CLJ_0213::*ermB*	Empty	TFTC*
	$CLJ\_0213_{1\text{-}648}$ (WT)	1.4×10^{-12}
	$CLJ\_0213_{40\text{-}648}$ (ΔTM1)	TFTC
	$CLJ\_0213_{99\text{-}648}$ (ΔTM1TM2)	TFTC
	$CLJ\_0213_{1\text{-}387}$ (ΔTM3)	4.7×10^{-13}
	$CLJ\_0213_{1\text{-}648}$ K175A	TFTC
	$CLJ\_0213_{28\text{-}648}$ (M28)	2.7×10^{-14}

*TFTC, tranconjugant colonies numbered too few to count

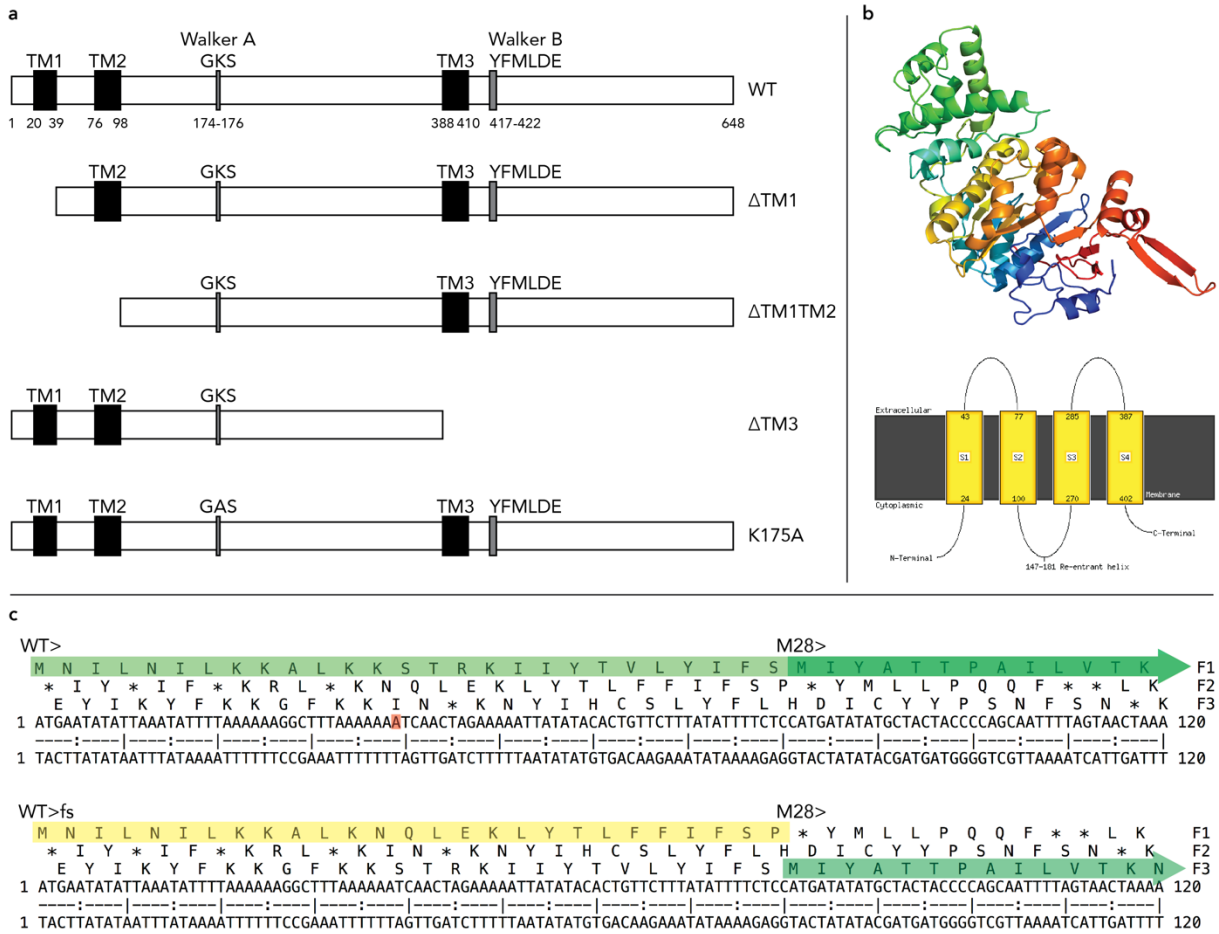

Figure 3. The gene product of CLJ_0213 is a putative ATPase with transmembrane helices. DNA and protein sequences of CLJ_0213 were analyzed with various bioinformatic servers as described in the text. (a) To evaluate the role of these CLJ_0213 domains in pCLJ conjugation, a point mutation was introduced to the ATP binding site (K175A) and truncations were made of each transmembrane helix region (ΔTMs). (b) Additional structural and topological predictions were generated with the Phyre2 server (32). (c) A frameshift mutation in the full-length CLJ_0213 ORF was observed in complementation constructs that could rescue the CLJ_0213::*ermB* conjugation defect. The nucleotide in red has been deleted from the bottom sequence, resulting in a premature stop codon. The alternative ORF that begins with M28 was subsequently evaluated. Three-frame translations were generated at the EMBOSS Sixpack webserver (33).

Discussion

The mobility of botulinum neurotoxins between strains is not only a topic of great evolutionary significance but also one with bearing on human and animal health and safety (13, 18, 34). In order to investigate the transfer of pCLJ, a 270 kb Group I *C. botulinum* plasmid encoding two BoNTs, we developed a plasmid transfer assay that relies on the *ermB*-RAM ClosTron and a *catP* derivative. While previous data have suggested conjugation as the mechanism of pCLJ transfer, neither the genes responsible nor their functions have been assigned to this plasmid. In the best-characterized clostridial conjugation locus—the *tcp* genes of *C. perfringens* plasmid pCW3—DNA transfer is achieved by an atypical relaxase and a membrane-spanning type IV secretion system (35). The *tcp* locus encodes the DNA-binding relaxase, two ATPases, and at least five membrane-associated proteins (23). Insertional inactivation of selected genes in the "conjugation region" of pCLJ revealed that CLJ_0213, a putative ATPase, is necessary for the plasmid's transfer to a representative *C. botulinum* recipient (Table 3). These data for the first time demonstrate that an ATPase within the conjugation region of a large neurotoxin-encoding *C. botulinum* plasmid is essential for its transfer, which is consistent with the mechanisms of plasmid conjugation in Gram-positive bacteria.

CLJ_0213 is annotated as an ATPase due to its Walker A and B motifs and its sequence is predicted to have at least three transmembrane helices (30). A fourth transmembrane helix is suggested by structural analysis on the Phyre2 server (Figure 3b) (32). The catalytic lysine residue in the Walker A motif is essential for rescuing the conjugation defect in CLJ_0213 (Figure 3a; Table 4). While deletion of one or both of the N-terminal transmembrane helices abrogated the ability of CLJ_0213 to restore conjugation to pCLJ in *trans*, the final transmembrane helix and subsequent C-terminal portion of the protein were apparently

dispensable for this function (Figure 3a; Table 4). The topology and stability of the CLJ_0213 constructs used in complementation, however, have not been determined.

The conjugation locus on pCLJ and other Group I *C. botulinum* plasmids is poorly defined. At present, the set of genes that may be involved in conjugation has been inferred from sequence gaps in plasmids that were not experimentally transferred (12, 24). This work provides an important first step toward characterizing the mechanism of conjugation in Group I *C. botulinum*. It is clear that CLJ_0213 plays a role in the transmission of BoNT-encoding plasmids in the clostridia, but we have not been able to determine whether any nearby genes are also essential. A recently-identified novel clostridial conjugation locus provides an instructive framework for defining the conjugal transfer apparatus. In *C. sordellii*, the pathogenicity locus (PaLoc) is encoded on plasmids of the pCS1 family, which are approximately 100 kb in size (36). These plasmids are now known to be transferred by conjugation, and the genes responsible are clustered in a locus of approximately 17 kb termed *cst* (37). Both putative ATPases in this locus, named CstB4 and CstD4 on the basis of their similarity to the *Agrobacterium tumefaciens* homologues VirB4 and VirD4, are essential for pCS1 conjugation (37). Vidor et al. note that the Group II *C. botulinum* plasmid pCLL has a locus homologous to *cst*; the bioinformatic analysis that produced this alignment did not detect a homologous locus on pCLJ (37). Although their identities are rather low, we have noted the ORFs on pCLJ that are most similar to *C. sordellii*'s major conjugation components (Table 5). CLJ_0235 and CLJ_0238—predicted to be a cell wall-associated hydrolase and a AAA ATPase, respectively—are strong candidates for future research on pCLJ conjugation.

Table 5. The pCLJ conjugation locus is likely not a contiguous operon. Open reading frames on *C. botulinum* Group I plasmid pCLJ with the greatest similarity to *C. sordellii cst* gene products, as determined by the blastp algorithm (38), are noted below.

C. sordellii ORF (Accession No.)	pCLJ ORF	Identity/Similarity	*C. botulinum* functional prediction
CstD4 (CEJ75489.1)	CLJ_0213	16%/28%	ATPase involved in conjugal plasmid transfer
CstB6 (CEJ75490.1)	CLJ_B0608*	11%/28%	Calcium-translocating P-type ATPase, PMCA-type
CstB4 (CEJ75493.1)	CLJ_0238	16%/36%	AAA ATPase
CstB1 (CEJ75496.1)	CLJ_0235	14%/25%	Putative cell wall-associated hydrolase
CstD2 (CEJ75502.1)	CLJ_B2125*	12%/25%	Two-component sensor kinase

C. sordellii protein sequences served as queries in blastp searches against *C. botulinum* Ba str. 657 with word size reduced to 2 and gap costs minimized.
*Chromosomal matches; no significant hits were found in plasmid sequences.

The identification of CLJ_0213 represents an important step forward in our understanding of the conjugation machinery of the Group I *C. botulinum* BoNT plasmids. The movement of *bont* genes within and outside of the species is a legitimate health and safety concern. While empirical evidence for *C. botulinum* plasmid conjugation is laborious to obtain and these experiments are restricted by Select Agent guidelines, the genetic contents of plasmids are typically widely available to the research community. CLJ_0213, then, may be suitable as a marker gene of *C. botulinum* conjugation. At present, there are uncharacterized CLJ_0213 homologues on at least a dozen BoNT-encoding *C. botulinum* plasmids in the NCBI database. A fuller investigation into the conjugative capacity of these plasmids and their ability to transfer toxigenicity to new and diverse clostridial strains is warranted.

Works Cited

1. Hill KK, Xie G, Foley BT, Smith TJ. Genetic diversity within the botulinum neurotoxin-producing bacteria and their neurotoxins. Toxicon. 2015;107(Pt A):2-8. Epub 2015/09/15. doi: 10.1016/j.toxicon.2015.09.011. PubMed PMID: 26368006.
2. Smith LD. The occurrence of *Clostridium botulinum* and *Clostridium tetani* in the soil of the United States. Health Lab Sci. 1978;15(2):74-80. Epub 1978/04/01. PubMed PMID: 355208.
3. Dabritz HA, Hill KK, Barash JR, Ticknor LO, Helma CH, Dover N, Payne JR, Arnon SS. Molecular epidemiology of infant botulism in California and elsewhere, 1976-2010. J Infect Dis. 2014;210(11):1711-22. Epub 2014/06/14. doi: 10.1093/infdis/jiu331. PubMed PMID: 24924163.
4. Espelund M, Klaveness D. Botulism outbreaks in natural environments - an update. Front Microbiol. 2014;5:287. Epub 2014/06/27. doi: 10.3389/fmicb.2014.00287. PubMed PMID: 24966853; PMCID: PMC4052663.
5. Rummel A. The long journey of botulinum neurotoxins into the synapse. Toxicon. 2015;107(Pt A):9-24. Epub 2015/09/13. doi: 10.1016/j.toxicon.2015.09.009. PubMed PMID: 26363288.
6. Chatham-Stephens K, Fleck-Derderian S, Johnson SD, Sobel J, Rao AK, Meaney-Delman D. Clinical Features of Foodborne and Wound Botulism: A Systematic Review of the Literature, 1932-2015. Clin Infect Dis. 2017;66(suppl_1):S11-S6. Epub 2018/01/03. doi: 10.1093/cid/cix811. PubMed PMID: 29293923.
7. Chalk CH, Benstead TJ, Keezer M. Medical treatment for botulism. The Cochrane database of systematic reviews. 2014(2):CD008123. Epub 2014/02/22. doi: 10.1002/14651858.CD008123.pub3. PubMed PMID: 24558013.
8. Koepke R, Sobel J, Arnon SS. Global occurrence of infant botulism, 1976-2006. Pediatrics. 2008;122(1):e73-82. Epub 2008/07/04. doi: 10.1542/peds.2007-1827. PubMed PMID: 18595978.
9. Peck MW, Stringer SC, Carter AT. *Clostridium botulinum* in the post-genomic era. Food Microbiol. 2011;28(2):183-91. Epub 2011/02/15. doi: 10.1016/j.fm.2010.03.005. PubMed PMID: 21315972.
10. Smith TJ, Hill KK, Foley BT, Detter JC, Munk AC, Bruce DC, Doggett NA, Smith LA, Marks JD, Xie G, Brettin TS. Analysis of the neurotoxin complex genes in *Clostridium botulinum* A1-A4 and B1 strains: BoNT/A3, /Ba4 and /B1 clusters are located within plasmids. PLoS One. 2007;2(12):e1271. Epub 2007/12/07. doi: 10.1371/journal.pone.0001271. PubMed PMID: 18060065; PMCID: PMC2092393.
11. Marshall KM, Bradshaw M, Pellett S, Johnson EA. Plasmid encoded neurotoxin genes in *Clostridium botulinum* serotype A subtypes. Biochem Biophys Res Commun. 2007;361(1):49-54. Epub 2007/07/31. doi: 10.1016/j.bbrc.2007.06.166. PubMed PMID: 17658467; PMCID: PMC2346372.
12. Marshall KM, Bradshaw M, Johnson EA. Conjugative botulinum neurotoxin-encoding plasmids in *Clostridium botulinum*. PLoS One. 2010;5(6):e11087. Epub 2010/06/17. doi: 10.1371/journal.pone.0011087. PubMed PMID: 20552020; PMCID: PMC2884020.
13. Nawrocki EM, Bradshaw M, Johnson EA. Botulinum neurotoxin-encoding plasmids can be conjugatively transferred to diverse clostridial strains. Scientific reports. 2018;8(1):3100. Epub 2018/02/17. doi: 10.1038/s41598-018-21342-9. PubMed PMID: 29449580; PMCID: PMC5814558.
14. Skarin H, Hafstrom T, Westerberg J, Segerman B. *Clostridium botulinum* group III: a group with dual identity shaped by plasmids, phages and mobile elements. BMC Genomics.

2011;12:185. Epub 2011/04/14. doi: 10.1186/1471-2164-12-185. PubMed PMID: 21486474; PMCID: PMC3098183.
15. Halpin JL, Hill K, Johnson SL, Bruce DC, Shirey TB, Dykes JK, Luquez C. Finished Whole-Genome Sequence of C*lostridium argentinense* Producing Botulinum Neurotoxin Type G. Genome Announc. 2017;5(21). Epub 2017/05/27. doi: 10.1128/genomeA.00380-17. PubMed PMID: 28546482; PMCID: PMC5477395.
16. Williamson CHD, Sahl JW, Smith TJ, Xie G, Foley BT, Smith LA, Fernandez RA, Lindstrom M, Korkeala H, Keim P, Foster J, Hill K. Comparative genomic analyses reveal broad diversity in botulinum-toxin-producing Clostridia. Bmc Genomics. 2016;17(1):180. Epub 2016/03/05. doi: ARTN 180
10.1186/s12864-016-2502-z. PubMed PMID: WOS:000371595000006; PMCID: PMC4778365.
17. Smith TJ, Hill KK, Raphael BH. Historical and current perspectives on *Clostridium botulinum* diversity. Res Microbiol. 2015;166(4):290-302. Epub 2014/10/15. doi: 10.1016/j.resmic.2014.09.007. PubMed PMID: 25312020.
18. Doxey AC, Mansfield MJ, Montecucco C. Discovery of novel bacterial toxins by genomics and computational biology. Toxicon. 2018;147:2-12. Epub 2018/02/14. doi: 10.1016/j.toxicon.2018.02.002. PubMed PMID: 29438679.
19. Wentz TG, Muruvanda T, Lomonaco S, Thirunavukkarasu N, Hoffmann M, Allard MW, Hodge DR, Pillai SP, Hammack TS, Brown EW, Sharma SK. Closed Genome Sequence of *Chryseobacterium piperi* Strain CTM^{T}/ATCC BAA-1782, a Gram-Negative Bacterium with Clostridial Neurotoxin-Like Coding Sequences. Genome Announcements. 2017;5(48). Epub 2017/12/02. doi: 10.1128/genomeA.01296-17. PubMed PMID: 29192076; PMCID: PMC5722062.
20. Zhang S, Lebreton F, Mansfield MJ, Miyashita SI, Zhang J, Schwartzman JA, Tao L, Masuyer G, Martinez-Carranza M, Stenmark P, Gilmore MS, Doxey AC, Dong M. Identification of a Botulinum Neurotoxin-like Toxin in a Commensal Strain of *Enterococcus faecium*. Cell host & microbe. 2018;23(2):169-76 e6. Epub 2018/02/06. doi: 10.1016/j.chom.2017.12.018. PubMed PMID: 29396040; PMCID: PMC5926203.
21. Zhang S, Masuyer G, Zhang J, Shen Y, Lundin D, Henriksson L, Miyashita SI, Martinez-Carranza M, Dong M, Stenmark P. Identification and characterization of a novel botulinum neurotoxin. Nature communications. 2017;8:14130. Epub 2017/08/05. doi: 10.1038/ncomms14130. PubMed PMID: 28770820; PMCID: PMC5543303.
22. Smillie C, Garcillan-Barcia MP, Francia MV, Rocha EP, de la Cruz F. Mobility of plasmids. Microbiol Mol Biol Rev. 2010;74(3):434-52. Epub 2010/09/02. doi: 10.1128/MMBR.00020-10. PubMed PMID: 20805406; PMCID: PMC2937521.
23. Wisniewski JA, Rood JI. The Tcp conjugation system of *Clostridium perfringens*. Plasmid. 2017;91:28-36. Epub 2017/03/14. doi: 10.1016/j.plasmid.2017.03.001. PubMed PMID: WOS:000403193700005.
24. Adams V, Li J, Wisniewski JA, Uzal FA, Moore RJ, McClane BA, Rood JI. Virulence Plasmids of Spore-Forming Bacteria. Microbiol Spectr. 2014;2(6). Epub 2015/06/25. doi: 10.1128/microbiolspec.PLAS-0024-2014. PubMed PMID: 26104459.
25. Heap JT, Pennington OJ, Cartman ST, Carter GP, Minton NP. The ClosTron: a universal gene knock-out system for the genus *Clostridium*. J Microbiol Methods. 2007;70(3):452-64. Epub 2007/07/31. doi: 10.1016/j.mimet.2007.05.021. PubMed PMID: 17658189.
26. Heap JT, Kuehne SA, Ehsaan M, Cartman ST, Cooksley CM, Scott JC, Minton NP. The ClosTron: Mutagenesis in *Clostridium* refined and streamlined. J Microbiol Methods. 2010;80(1):49-55. Epub 2009/11/07. doi: 10.1016/j.mimet.2009.10.018. PubMed PMID: 19891996.

27. Bradshaw M, Marshall KM, Heap JT, Tepp WH, Minton NP, Johnson EA. Construction of a nontoxigenic *Clostridium botulinum* strain for food challenge studies. Appl Environ Microbiol. 2010;76(2):387-93. Epub 2009/11/26. doi: 10.1128/AEM.02005-09. PubMed PMID: 19933346; PMCID: PMC2805223.
28. Heap JT, Pennington OJ, Cartman ST, Minton NP. A modular system for *Clostridium* shuttle plasmids. J Microbiol Methods. 2009;78(1):79-85. Epub 2009/05/19. doi: 10.1016/j.mimet.2009.05.004. PubMed PMID: 19445976.
29. Girbal L, Mortier-Barriere I, Raynaud F, Rouanet C, Croux C, Soucaille P. Development of a sensitive gene expression reporter system and an inducible promoter-repressor system for *Clostridium acetobutylicum*. Appl Environ Microbiol. 2003;69(8):4985-8. Epub 2003/08/07. PubMed PMID: 12902297; PMCID: PMC169153.
30. Krogh A, Larsson B, von Heijne G, Sonnhammer EL. Predicting transmembrane protein topology with a hidden Markov model: application to complete genomes. J Mol Biol. 2001;305(3):567-80. Epub 2001/01/12. doi: 10.1006/jmbi.2000.4315. PubMed PMID: 11152613.
31. Hu J, Li Y, Zhang Y, Yu DJ. ATPbind: Accurate Protein-ATP Binding Site Prediction by Combining Sequence-Profiling and Structure-Based Comparisons. J Chem Inf Model. 2018;58(2):501-10. Epub 2018/01/24. doi: 10.1021/acs.jcim.7b00397. PubMed PMID: 29361215; PMCID: PMC5963530.
32. Kelley LA, Sternberg MJ. Protein structure prediction on the Web: a case study using the Phyre server. Nat Protoc. 2009;4(3):363-71. Epub 2009/02/28. doi: 10.1038/nprot.2009.2. PubMed PMID: 19247286.
33. McWilliam H, Li W, Uludag M, Squizzato S, Park YM, Buso N, Cowley AP, Lopez R. Analysis Tool Web Services from the EMBL-EBI. Nucleic Acids Res. 2013;41(Web Server issue):W597-600. Epub 2013/05/15. doi: 10.1093/nar/gkt376. PubMed PMID: 23671338; PMCID: PMC3692137.
34. Montecucco C, Rasotto MB. On botulinum neurotoxin variability. MBio. 2015;6(1). Epub 2015/01/08. doi: 10.1128/mBio.02131-14. PubMed PMID: 25564463; PMCID: PMC4313909.
35. Grohmann E, Keller W, Muth G. Mechanisms of Conjugative Transfer and Type IV Secretion-Mediated Effector Transport in Gram-Positive Bacteria. In: Backert S, Grohmann E, editors. Type IV Secretion in Gram-Negative and Gram-Positive Bacteria. Cham: Springer International Publishing; 2017. p. 115-41.
36. Couchman EC, Browne HP, Dunn M, Lawley TD, Songer JG, Hall V, Petrovska L, Vidor C, Awad M, Lyras D, Fairweather NF. *Clostridium sordellii* genome analysis reveals plasmid localized toxin genes encoded within pathogenicity loci. BMC Genomics. 2015;16:392. Epub 2015/05/20. doi: 10.1186/s12864-015-1613-2. PubMed PMID: 25981746; PMCID: PMC4434542.
37. Vidor CJ, Watts TD, Adams V, Bulach D, Couchman E, Rood JI, Fairweather NF, Awad M, Lyras D. *Clostridium sordellii* Pathogenicity Locus Plasmid pCS1-1 Encodes a Novel Clostridial Conjugation Locus. MBio. 2018;9(1). Epub 2018/01/18. doi: 10.1128/mBio.01761-17. PubMed PMID: 29339424; PMCID: PMC5770547.
38. Altschul SF, Madden TL, Schaffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. 1997;25(17):3389-402. Epub 1997/09/01. PubMed PMID: 9254694; PMCID: PMC146917.

Chapter 3: Molecular manipulations in *C. botulinum*

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Author Contributions

E.M.N. and M.B. generated the BoNT/EA hybrid variants and the modified *C. botulinum* strain used to isolate BoNT/FA. E.M.N. designed and conducted recombineering and CRISPR-Cas9 experiments and drafted this summary chapter. E.A.J. assisted in the supervision and critique of the research.

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Additional Information

The authors declare that they have no conflicts of interest.

Abstract

The various serotypes of botulinum neurotoxin (BoNT) share a common domain structure, consisting of a 50 kDa light chain (LC) and a 100 kDa heavy chain (HC). The genomic loci of *bont* clusters are well characterized, and their sequences are known in many isolates. The various BoNT serotypes and subtypes all cause the flaccid paralysis typical of botulism, but they vary in characteristics including potency, duration of action, in vivo symptoms, species specificity, and cell entry kinetics. Functional studies on these toxins are limited by their classification as Select Agents and the accompanying regulatory restrictions. Recombinant expression of BoNTs, the creation of mutated BoNTs, and the introduction of mutations into the *C. botulinum* genome are restricted experiments in the United States, with few labs having the requisite approval. This as well as the genetics and physiology of *C. botulinum* have led to a relative lack in genetic methods available for the organism. As genetic methods in clostridia and other bacteria are advancing, new technologies are emerging that will allow more rapid genome editing in *C. botulinum*. This chapter analyzes several avenues of genetic manipulation of *C. botulinum* and BoNTs.

3A: Generation of hybrid botulinum neurotoxins

Introduction

Clostridium botulinum, an anaerobic spore-forming bacterium, produces botulinum neurotoxin (BoNT). Intoxication with BoNT causes botulism, which leads to flaccid paralysis and eventual death if untreated. BoNTs are 150 kDa proteins consisting of a 100 kDa heavy chain (HC) and a 50 kDa light chain (LC). The light chain is the catalytically active portion, a zinc endopeptidase that specifically cleaves components of the SNARE machinery in vertebrate neurons (1-5). As has been abundantly reviewed in the literature, the heavy chain is responsible for receptor binding, entry into neurons, and translocation into the cytosol (6-8).

BoNT proteins exist in at least seven serotypes, designated BoNT/A through BoNT/G, defined by virtue of their neutralization with homologous antisera. Serotypes are further divided into subtypes, designated with a numeral (e.g. BoNT/A1), on the basis of their amino acid sequence and neutralization with homologous antisera (9). The sequence variation of subtypes within a serotype can make botulism detection and treatment more challenging, as it may alter antibody binding and neutralization (10).

All BoNTs target the SNARE machinery, but the serotypes have different substrates. BoNT/A and /E cleave SNAP-25, a SNARE complex protein on the plasma membrane, at distinct sites. BoNT/C cleaves SNAP-25 and syntaxin, which is also membrane-associated. BoNT/B, /D, /F, and /G each cleave VAMP (a vesicle-associated SNARE protein, also known as synaptobrevin) at distinct sites. Furthermore, the serotypes have distinct durations of activity and produce different symptoms in mouse bioassays. For instance, despite targeting the same protein substrate, BoNT/A persists much longer than BoNT/E does in cell culture (11, 12), and paralysis after local intramuscular injection similarly persists for much longer (12, 13). Several

BoNT/A subtypes also display diverse potency, duration of action, and cell entry kinetics, and they cause different symptoms in the mouse bioassay (14, 15).

Despite the differences, the fact that all BoNT serotypes share a common structure has sparked research into the modularity of the HC and LC. In Group III *C. botulinum* strains, the BoNT/C and /D genes are carried on bacteriophages, and the finding of numerous mosaic BoNT/CD and /DC toxins suggest recombination events between the two serotypes (16). Recent genome sequences from Group I *C. botulinum* indicate other natural hybrid BoNT variants (17). The resulting hybrid toxins have distinct immunological and biological properties, which may hold significance for the development of novel BoNT-based therapeutics and indications, for treatment of clinical botulism cases, for detection methods of BoNTs in clinical and environmental samples, and for the assessment of biothreats posed by BoNTs and the development of countermeasures. An ability to design and recombinantly construct hybrid BoNTs enables their functional characterization, but so far only few such hybrids have been constructed due to technical and regulatory difficulties. As both the binding domain and the active site demonstrate specificity, BoNTs may be manipulated along multiple axes for therapeutic use (18).

In order to characterize novel toxin variants and evaluate their therapeutic potential, our laboratory has developed the first *C. botulinum*-based system for the recombinant expression of BoNTs. Using a modified *C. botulinum* strain avoids certain pitfalls of other recombinant expression platforms, such as the lack of stabilizing non-toxic complex proteins, the need for codon optimization in *E. coli,* and the lack of proper posttranslational processing in *E. coli* and eukaryotic systems (19). *E. coli* and *P. pastoris* are the most common expression systems for producing botulism toxoids (e.g. for use in vaccines), but other protocols, including a baculovirus-based vector expressed in insect cells, are also available (20, 21). Our lab created a

C. botulinum derivative in which the native BoNT is not expressed to serve as a recombinant expression host. This strain, Hall A-*hyper*/tox$^-$, was rendered nontoxigenic by insertion of a ClosTron module (Em^R) in its native *bont/a* locus. This strain yields high level expression of recombinant BoNT/A subtypes. In addition, the nontoxic complex proteins NTNH, HA50, HA33, HA20, and HA17 are unaltered in Hall A-*hyper*/tox$^-$ and can form complexes with recombinantly expressed BoNTs to mimic expression, posttranslational processing, and stability of the native toxin (19).

In this work, we investigated the utility of Hall A-*hyper*/tox$^-$ in expressing a chimeric BoNT/EA protein. The broad goal of this study was to design an atoxic BoNT molecule to carry a nanobody (AA1) directed against the BoNT/A light chain (LC) into neurons. The nanobody is a reactive antibody derivative that binds to and inhibits the activity of the BoNT/A1 LC, allowing us to investigate the intracellular inhibition of BoNT toxicity. Attaching the nanobody to catalytically inactive BoNT proteins with tropism for motor neurons is a strategy for in vivo treatment of intoxication with botulinum neurotoxin or delivery of other bioactive molecules (21-23). The BoNT/E LC was chosen for the carrier to avoid binding of the nanobody to the carrier itself, as would occur if BoNT/A LC was used, and because of the short intracellular half-life of BoNT/E LC, to avoid accumulation and long persistence of the carrier in neurons (11, 24). The BoNT/A2 HC was chosen to allow entry into the same neurons as BoNT/A, against which the potential therapy is directed, and because BoNT/A2 has been shown to enter neurons faster and more efficiently than other BoNT/A subtypes (14).

Methods

Bacterial strains and culture conditions

Clostridium botulinum isolates were maintained in TPGY broth (50 g/l trypticase peptone, 5 g/l Bacto peptone, 4 g/l dextrose, 20 g/l yeast extract, 1 g/l cysteine-HCl, pH 7.4) for liquid culture and TYG agar (30 g/l Bacto tryptone, 20 g/l yeast extract, 1 g/l sodium thioglycolate, 15 g/l agar, pH 7.4) for solid culture. *Escherichia coli* strains were maintained in 2 × YT media (16 g/l Bacto tryptone, 10 g/l yeast extract, 5 g/l sodium chloride, 15 g/l agar, pH 7.0). Frozen stocks were made from liquid cultures supplemented with 20% glycerol and were stored at -80°C. Antibiotics were used as follows: ampicillin at 100 µg/ml; chloramphenicol at 12.5 µg/ml in broth and 25 µg/ml in agar; erythromycin at 50 µg/ml; cycloserine at 250 µg/ml; and thiamphenicol at 15 µg/ml. All chemicals and media components were purchased from Becton Dickinson Microbiology Systems (Sparks, MD) and Sigma-Aldrich (St. Louis, MO) unless otherwise noted.

E. coli strains were grown aerobically at 37°C, shaking at 225 rpm where appropriate. Chemically competent cells of strains DH10B (for routine cloning) and CA434 (for conjugation into *C. botulinum*) were typically prepared in-house (25). Clostridial strains were grown under anaerobic conditions. Glass culture tubes were flushed with nitrogen gas and sealed with butyl rubber stoppers (Bellco Glass, Vineland, NJ) before sterilizing. All clostridial experiments were performed in an anaerobic chamber (Forma Anaerobic System, Marietta, OH) with an initial gas mixture of 80% N_2, 10% CO_2, and 10% H_2. Resazurin was added to solid media at 2 µg/ml, and agar plates were prereduced by overnight incubation in the anaerobic chamber.

Table 1. Strains, plasmids, and oligonucleotides used in this study. All oligonucleotides were purchased from Integrated DNA Technologies (Coralville, IA). Restriction sites are underlined.

Name	Description	Reference
C. botulinum Alaska E	Group II *C. botulinum*, BoNT/E3 producer	(26)
C. botulinum Kyoto F	Group I *C. botulinum*, BoNT/A2 producer	(27)
C. botulinum Hall A-*hyper*/tox$^-$	Group I C. botulinum, *bont/a1* gene inactivated by ClosTron insertion	(28)
E. coli CA434	Plasmid donor strain	(29, 30)
pMTL80000 series	*E. coli-Clostridium* shuttle vectors	(29)
Nanobody fwd	GCCATATGCATCATCATCATCATCATGCTG	
Nanobody rev	CTATTAATTTTTGGACCACCACCTCCACCAGAAG	
E3LC fwd	CTGGTGGAGGTGGTGGTCCAAAAATTAATAGTTTTAATTATAATGATCCTGTTAATGATAGAAC	
E3LC rev	GGTATTATCCCTCTTACACAAAATCTAATGATTTTTTTTACTAGTCCTC	
E3HCN rev	CTCAATATAGAGGTATTAACAATATTCTTAAAGAATTTATTAAAATATGAAATTAAAATTTTATCATCTG	
A2HC fwd	GAGGACTAGTAAAAAAAATCATTAGATTTTGTGTAAGAGGGATAATACC	
A2HCC fwd	CAGATGATAAAATTTTAATTTCATATTTTAATAAATTCTTTAAGAATATTGTTAATACCTCTATATTGAG	
A2HC rev	GGCGGCGCGCCATAAAAATAAGAAGCCTGCAAATGCAGG	
E3 631-673 QuikChange	GCTCTTACATTAATGCATGCATTAATACATTCATTACATGGAC	
E3 1030-1086 QuikChange	GCAACTAAATTTCAAGTTAAATGTGCAGAAACTTTTATTGGACAGTATAAATACTTC	
E3_513F	GCCAAGCAATCACGGTTTTGGATC	
E3_815R	GTATAGATATCATTGTACTGAGCAACAG	
E3_931F	GATGCTAGCGGAATTTATTCGG	
E3_1178R	CTAGGATTTAAATTTGCATTCTGTCC	
E3_1189F	CCAATTACAGGTAGAGGACTAG	
E3_1682R	GCTGTATCAATTGAAGAGGTGAG	
E3_1714F	GTAAGCTGGATACAACAAGTGTTAGTAG	
E3_2185R	CCTCTAAAGTATAACTATTATACTTAG	
E3_2252F	CTATAGCAATGAATAATATAGACAGG	
A2_2791F	GCTATTGTATATAATAGTATGTATG	

BoNT/EA hybrid toxin assembly

Genomic DNA was extracted from overnight cultures of *C. botulinum* strains Alaska E and Kyoto F (Table 1) according to the ChargeSwitch gDNA protocol (Life Technologies, Carlsbad, CA) and stored at -20°C for future use. Oligonucleotide primers (Table 1) were designed to amplify portions of the genomic *bont* genes, varying in length and appended with additional nucleotides to facilitate overlap assembly of PCR products (Figure 1). Reactions were performed with Phusion High-Fidelity PCR Master Mix (New England Biolabs, Ipswich, MA) on an Applied Biosystems 9600 thermocycler. Mutations to the BoNT catalytic site were introduced with the QuikChange Mutagenesis kit (Agilent Biotechnologies, Santa Clara, CA) to render the molecule nontoxigenic (Table 1). Component fragments of the hybrid toxins were joined by overlap PCR using the outermost primers and purified from agarose gels as necessary using the GeneTel kit (Madison, WI). Full-length hybrid toxin genes were cloned into a blunted pJET1.2 fragment using the CloneJET kit (ThermoFisher Scientific, Waltham, MA) and verified with Sanger sequencing using the pJET1.2 forward and reverse primers and the sequencing primers given in Table 1. All DNA sequences were verified using the given primers in a BigDye Terminator v3.1 reaction (Applied Biosystems, Foster City, CA), cleaned up with magnetic beads, and analyzed at the University of Wisconsin-Madison Biotechnology Center. Constructs with the correct nucleotide sequence were subcloned into vectors from the pMTL modular series (29) for expression in *C. botulinum*. Toxins were placed under the control of clostridial promoter sequences derived from the thiolase (P_{thl}) or ferredoxin (P_{fdx}) gene to drive constitutive expression. The promoter modules were paired with Gram-positive replication sequences from *C. botulinum* (pBP1) or *C. butyricum* (pCB102) in various combinations to assess plasmid stability (Figure 2).

Recombinant toxin expression and analysis

Expression vectors were transformed into *E. coli* CA434 to prepare for clostridial matings (29, 30). The *C. botulinum* strain Hall A-*hyper*/tox$^-$, engineered in our laboratory for recombinant toxin expression, was mated with the *E. coli* plasmid donor strain as previously described (28). Transconjugants were recovered on agar containing cycloserine to select against *E. coli* and thiamphenicol to select for the hybrid toxin expression vector. Colonies were restreaked to purify and selected isolates were grown for 96 hours in toxin production medium at 37°C. Whole culture lysates were sampled every 24 hours into NuPAGE SDS sample buffer (Life Technologies, Waltham, MA), heated to 80°C for 5 minutes, and stored at -20°C until processed. The lysates were reduced with betamercaptoethanol and analyzed by SDS-PAGE. Proteins were separated by electrophoresis at 150 V through 4-12% Bis-Tris NuPAGE Novex gels in morpholineethane sulfonic acid (MES) running buffer (ThermoFisher Scientific, Waltham, MA). The gels were stained with Coomassie dye or transferred to PVDF membranes using a semi-dry transfer protocol (1 h at 0.2 A per gel). The membranes were then probed with a polyclonal affinity-purified rabbit antibody raised in-house against the BoNT/A holotoxin. Images were developed with the chemiluminescent PhosphaGLO alkaline phosphatase substrate (KPL, Gaithersburg, MD). A representative isolate was chosen for toxin extraction by ammonium sulfate precipitation, which was achieved by gradual addition of ammonium sulfate to 60% saturation at ambient temperature. The suspension was stored at 4°C for further processing.

Results

Generation of hybrid botulinum neurotoxins

Four chimeric neurotoxin constructs were designed to investigate the emergent properties of a BoNT/A-BoNT/E hybrid toxin (Figure 1). First, the catalytically inactive (23, 31) light chain of BoNT/E3 and the heavy chain of BoNT/A2 were combined, with the AA1 nanobody fused to the N-terminus of the LC. Because the N-terminus of the BoNT HC encodes a structural "belt" region that wraps around the LC, and it is unknown whether the BoNT/A "belt" can wrap around the BoNT/E LC, the BoNT/E3 LC was extended the through the belt region. Since it is also unknown whether a BoNT/EA LC-HC chimera can form a structurally stable holotoxin, a second chimera was created in which only the HC receptor binding domain of BoNT/E was replaced with that of BoNT/A2 (Figure 1). The receptor binding domain and translocation domain in the HC are structurally independent domains joined by a "single loop junction" and structural modeling indicates that replacing the receptor binding domain would not structurally disturb the BoNT/E LC-HCN. In order to be able to examine cell entry in a quantitative manner, the same two constructs were also created using an enzymatically active BoNT/E3 LC, so that cell entry could be evaluated by calculating SNAP-25 cleavage (Figure 1).

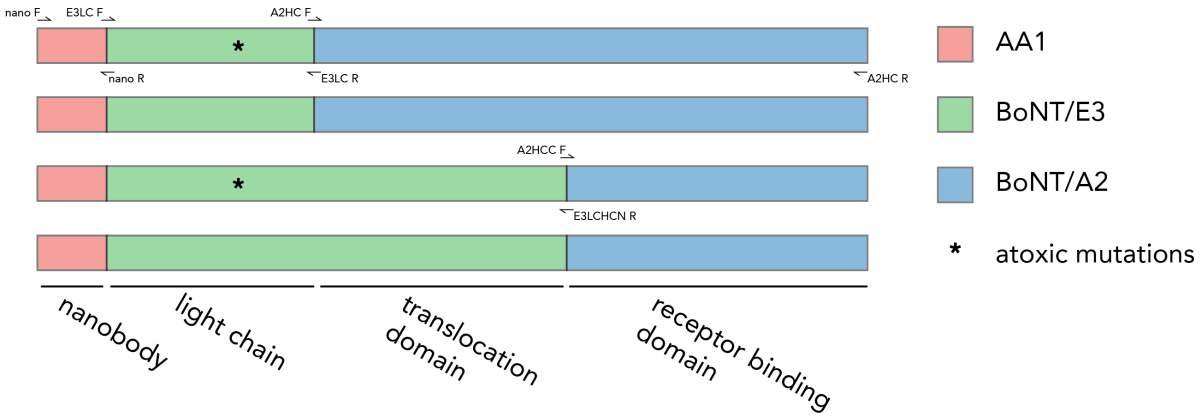

Figure 1. Hybrid BoNTs combine light and heavy chains from assorted serotypes. Genomic DNA preparations from the Alaska E and Kyoto A strains were used as templates for PCR. Primers were designed with flanking sequences homologous to the overlapping fragments. The three amplicons were joined in an overlap PCR using the outermost primer pair. Site-directed mutagenesis reactions were used to introduce the three amino acid substitutions that render the BoNT inactive. See Table 1 for all oligonucleotide primer sequences.

Expression of recombinant BoNT/EA constructs

The clostridial expression hosts harboring the hybrid toxin constructs were grown anaerobically in toxin production medium for 4 days and sampled every 24 h. Whole culture lysates were examined by SDS-PAGE and Western blot using a primary polyclonal antibody raised against the BoNT/A holotoxin (Figure 2). Considering all BoNT/EA hybrids tested, maximal expression was achieved in the pMTL82153 vector carrying the nano-E3LCHCN-A2HCC construct at 24 h (Figure 2). There was no substantial increase in signal in other expression constructs over the remaining time course, and therefore only the pMTL82153::nano-E3LCHCN-A2HCC clone was chosen for further analysis. After increasing the culture volume and attempting to precipitate the toxin with the addition of ammonium sulfate, it was determined that the yield of the BoNT/EA hybrid was not suitable for large-scale purification. These data indicate either that the *C. botulinum* Hall A-*hyper*/tox$^-$ expression host is not a suitable expression host for these chimeric constructs, or that the chimeric proteins were not structurally stable. Due to a lapse in funding for this project, no further experiments were conducted to investigate the underlying cause of failed expression.

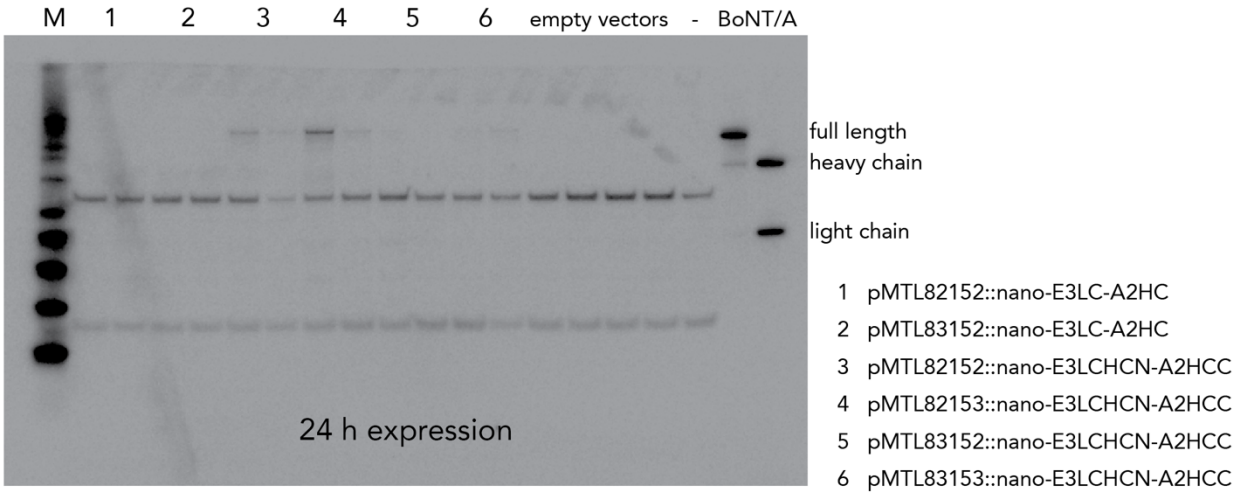

Figure 2. BoNT/EA hybrids are poorly expressed in the Hall A-*hyper*/tox$^-$ recombinant host. Following assembly by overlap PCR, the BoNT/EA hybrid neurotoxins were attached to constitutive clostridial promoters and ligated into the pMTL modular vector series. Expression was greatest at 24 hours by the pMTL82153::nano-E3LCHCN-A2HCC construct as determined by immunoblot. M, Magic Mark Protein Standard; -, Hall A-*hyper*/tox$^-$.

Discussion

Although fusing the heavy and light chains of two toxin serotypes was accomplished with ease in *E. coli* cloning hosts, the expression of the resultant BoNT/EA hybrid in *C. botulinum* Hall A-*hyper*/tox$^-$ was inefficient (Figure 2). The same *C. botulinum* strain has been used to purify recombinant BoNT/A4 to large quantities (19). It is possible that the BoNT/EA hybrid is poorly expressed in *C. botulinum* Hall A-*hyper*/tox$^-$, or that it is rapidly degraded in the expression strain. This could be due to an inability of the chimeric toxin to associate with the protective and stabilizing nontoxic complex proteins in this expression host. There is precedent for the expression of hybrid toxins in this system, although the novel molecules generated therein were fusions of various BoNT/A subtypes (e.g. BoNT/A1A3) (32). The case may be that the introduction of a BoNT segment from a different physiological Group of *C. botulinum*, such as BoNT/E from Group II, is poorly tolerated. Note that the toxin complex flanking *bont/e* consists of *orfX* genes, while the complex produced in Hall A-*hyper* consists of *ha* genes (33). Heterologously expressed toxins can associate and coimmunoprecipitate with their noncognate clusters, but the process by which they assemble intracellularly is not clear (34). Intriguingly, several BoNT hybrids have been successfully purified from *E. coli* expression strains, including one case in which the light chain of BoNT/E was fused to the entirety of the BoNT/A protein (35). This indicates that a structurally stable BoNT/AE hybrid can be produced, although in the case of this study several amino acid residues have been added at the fusion site and tags added for purification. Recombinant BoNTs produced in *E. coli* will not undergo all necessary posttranslational modifications to produce active BoNTs, and while some of these modifications, such as the cleavage between LC and HC to produce the dichain molecule, can be reproduced in vitro, others are not yet explored. It is therefore important to further develop endogenous expression systems for BoNTs to be able to investigate properties of novel toxins, chimeric

toxins, and the role of structural motifs or specific amino acids in BoNTs. While this study had to be ended due to the discontinuation of funding, it shows that the production and purification of hybrid toxins or heterologous BoNT serotypes in *C. botulinum* expression hosts are topics that merit additional study.

3B: Creation of a modified *C. botulinum* strain for isolation of a novel BoNT

Summary

Similar genetic and molecular cloning techniques were utilized to isolate, express, and characterize a naturally-occurring BoNT hybrid. A novel neurotoxin possessing genetic similarity to BoNT/F and BoNT/A was identified in a dual-toxin-producing *C. botulinum* strain (renamed CDC69016) implicated in a case of infant botulism (17, 36). Because this strain produces two neurotoxins—the novel hybrid and BoNT/B2—it is difficult to purify a single toxin to homogeneity. In an attempt to circumvent this barrier, we decided to insertionally inactivate the *bont/b2* gene using the ClosTron method of mutagenesis. Abolishing the expression of the "major" BoNT/B2 toxin would allow us to target the novel BoNT/FA for full characterization and purification. The following results are adapted from a manuscript describing these efforts (37).

Methods

The major toxin in the dual-BoNT-producing strain CDC69016 was inactivated via ClosTron insertion in order to selectively purify a second toxin first described as a new serotype (17, 36) and confirmed by our laboratory as a novel hybrid toxin BoNT/FA (37). A construct targeting the *bont/b2* gene was designed using the Perutka algorithm (see clostron.com) to insert a mobile group II intron between nucleotides 380 and 381 on the sense strand of the gene, disrupting BoNT/B2 expression (38, 39). The retargeted ClosTron vector was transformed into chemically competent *E. coli* CA434 cells and transferred by conjugation to *C. botulinum* CDC69016 (29, 30, 40). Transformants were recovered on media selective for the Tm^R ClosTron vector, then replica plated on media selective for the Em^R retrotransposition-activated marker (RAM). Once the intron's insertion into the *bont/b2* gene was verified by PCR (Table 2), isolates were restreaked to screen for loss of the targeting vector.

To confirm that ClosTron mutants contained only a single insertion, genomic DNA was isolated from *C. botulinum* CDC69016 and its mutant derivatives, and 5 µg of each gDNA preparation was digested with HindIII. The HindIII digests were analyzed by Southern hybridization using a ^{32}P-radiolabeled probe for the *ermBP* gene (Table 2). Membranes were exposed to autoradiography film and imaged as previously described (41).

Table 2. Strains, plasmids, and oligonucleotides used in this work. All oligonucleotides were purchased from Integrated DNA Technologies (Coralville, IA).

Name	Description	Reference
C. botulinum Hall A-*hyper*	Group I *C. botulinum*, BoNT/A1 producer	(42)
C. botulinum Hall A-*hyper*/tox$^-$	Group I *C. botulinum*, *bont/a1* gene inactivated by ClosTron insertion	(28)
C. botulinum CDC69016	Group I *C. botulinum*, dual toxin producer, isolated as IBCA10-7060	(36, 37)
C. botulinum CDC-A3	Group I *C. botulinum*, BoNT/A3 producer	(43)
E. coli CA434	Plasmid donor strain	(29, 30)
pMTL007C-E2::Cbo:bontbvB-381s	ClosTron vector targeting *C. botulinum bont/b2* gene for insertional inactivation	(38, 40)
bont/b2 fwd	CAAACAATGATCAAGTTATTTAATAG	
bont/b2 rev	TCATTTAAAACTGGCCCAGG	
ermBP fwd	ATGAACAAAAATATAAAATATTCTCAAAAC	
ermBP rev	TTATTTCCTCCCGTTAAATAATAGATAACG	

Results

Expression of the BoNT/B2 in strain CDC69016 can be eliminated while maintaining expression of BoNT/FA. To facilitate purification and characterization of BoNT/FA from the dual-toxin-producing strain CDC69016, production of BoNT/B2 was genetically eliminated in this strain by inactivation of the *bont/b2* gene using the ClosTron mutagenesis system (40, 44). As shown in Figure 3a, insertion of the ClosTron module near the 5' end of the gene prohibits its expression and generates a PCR amplicon 1.8 kb larger than that of the wild-type using the same forward and reverse primer pair. Insertion at the proper locus was confirmed by PCR (Figure 3b) and by Southern hybridization, demonstrating that no ClosTron inserts were found elsewhere in the genome (Figure 3c-d). The resultant strain was designated CDC69016/B2tox$^-$. The lack of active BoNT/B2 was subsequently demonstrated by Western blot and by neutralization studies in neuronal cell models and in the mouse bioassay (37).

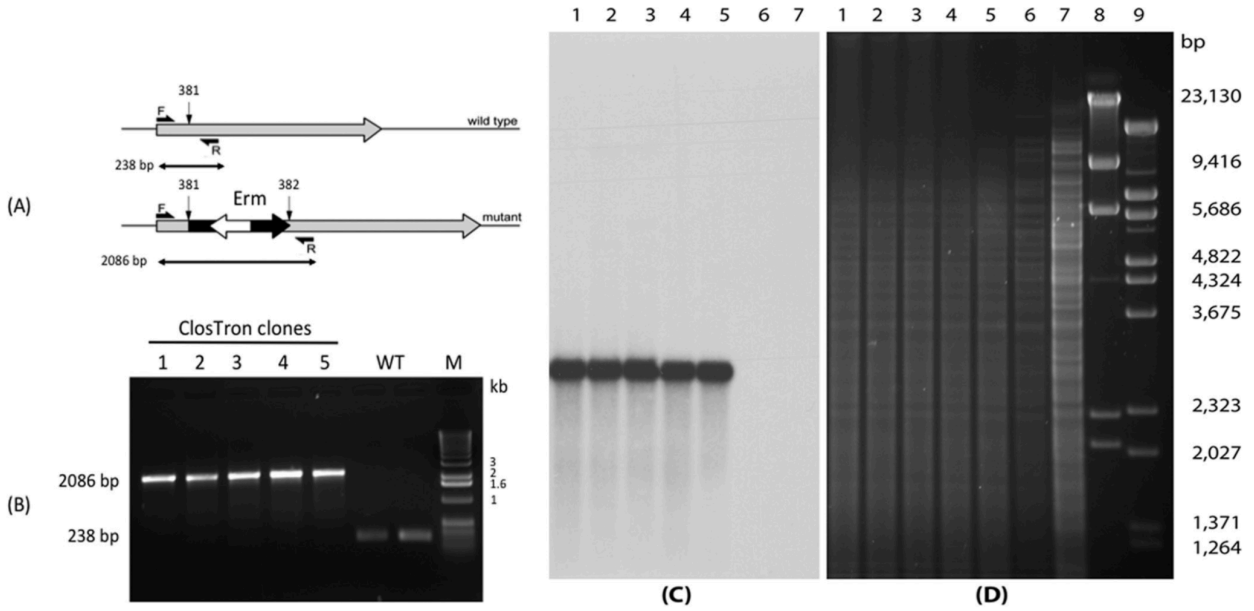

Figure 3. Genetic disruption of the *bont/b2* gene in strain CDC69016. (A) Schematic presentation of the wild-type (top) and mutated (bottom) botulinum neurotoxin B2 gene. The group II intron is shown as a black wide arrow inserted on the sense strand of the toxin gene (gray arrow) between nucleotides 381 and 382 as indicated by the vertical black arrows. The white arrow inside the intron element in the opposite orientation to the intron and toxin gene is a retrotransposition-activated erythromycin (RAM-Erm) resistance gene. The locations of the forward (F) and reverse (R) PCR primers are shown with horizontal arrows on either side of the intron insertion site. The expected sizes of the PCR products are 238 bp for the wild-type gene (WT) and 2,086 bp for the inactivated *bont/b2* gene. (B) PCR products of five putative mutant clones (clones 1 to 5) and two samples of the WT strain. The positions of molecular size markers (M) (Track-it DNA ladder [Life Technologies]) in base pairs are shown to the right of the gel. (C and D) Southern hybridization with the intron probe (erythromycin gene) (C) and ethidium bromide-stained 1% agarose gel of genomic DNA digested with restriction enzyme HindIII (D). Lanes 1 to 5, five putative *bont/b2* mutant clones; lane 6, wild-type CDC69016 strain; lane 7, *C. botulinum* strain CDC-A3 as a negative control; lanes 8 and 9, DNA markers (NEB, Ipswich, MA). The sizes of the DNA markers (in base pairs) are indicated to the right of the gel. Reproduced from Pellett et al. 2016 (37).

Discussion

There are a number of advantages to purifying BoNTs directly from the *C. botulinum* strain in which they originate, in particular to investigate the properties of novel or unexplored toxins. Although one might experimentally adjust the culture conditions for maximal expression, the toxin gene need not be codon optimized and the BoNT is free to associate with its cognate complex proteins. The purification of BoNT/FA from its native dual-toxin-producing *C. botulinum* strain is a template for future research of this type. A short discussion section from the relevant manuscript (37) is reprinted below.

> Purification and subsequent characterization of toxins produced by dual-toxin producers are essential to gain further understanding of this occurrence. However, purification is usually difficult in dual-toxin producers, since the BoNTs are similar in structure, molecular weight, and ionic properties. Affinity chromatography using antibodies specific for different serotypes can be used to purify relatively low quantities of BoNTs, and other approaches such as selective binding to receptor proteins or SNARE (soluble *N*-ethylmaleimide-sensitive fusion factor attachment protein receptors) substrates is theoretically feasible, but such approaches would require extensive work.

In summary, the challenges of heterologous expression of BoNTs are often surmountable, but the logical preference is to engineer a means of isolating a toxin from its native host. In bivalent *C. botulinum* strains, the ClosTron method of insertionally inactivating an undesired *bont* gene has proven sufficient (Figure 3). In more challenging strain backgrounds, as when there are additional undesired genes expressed, selective toxin purification will require alternative strategies at the DNA or protein level. Here, the ClosTron strategy enabled purification and antigenic and biologic characterization of a novel BoNT, which had originally been described as a new serotype and potentially serious biothreat with no available countermeasures (17, 36). Our laboratory in conjunction with the Centers for Disease Control, Atlanta, was able to demonstrate neutralization of this toxin by existing antisera and reclassify the novel toxin as a BoNT/F5A hybrid (45, 46).

3C: Evaluation of recombineering and CRISPR/Cas in *C. botulinum*

Summary

Research into all aspects of *C. botulinum* is often stalled by our inability to make gene deletions in the species. In order to expand the repertoire of tools for use in the species, and to identify additional genes necessary for plasmid transfer, we pursued genetic techniques including single-stranded DNA recombineering and CRISPR-Cas9 genome editing, both of which have been employed successfully in other Gram-positive organisms.

The implementation of recombineering technology in a new organism requires careful oligonucleotide design and a functional RecT protein under the control of an inducible promoter. RecT, a relative of the lambda phage protein Redβ, anneals to single-stranded DNA and promotes strand exchange by aiding in the pairing of complementary fragments (47). Tight control of the recombinase protein is necessary, as extended expression can cause cell lethality (48). Recombinases from a variety of bacterial species, including Gram-negatives and Gram-positives alike, were able to catalyze recombineering in *E. coli*, suggesting the proteins have a broad host range (49). Still, there are instances in which RecT homologues from species within the same genus have adverse effects on recombineering efficiency (50).

The components required for a CRISPR-Cas9 genome editing platform are also minimal (a nuclease, a targeting construct, and an editing template) and the editable sequences are plentiful (51). At times, the editing template is provided as a recombineering oligonucleotide. On its own, the efficiency of recombineering is sometimes too low to be practical, especially if the technique is to be used to create insertions or deletions. Coupled with the strong selective pressure of CRISPR-Cas9, recombineering becomes a significantly more powerful tool. The two techniques were investigated in parallel for this project.

Methods

Recombineering and CRISPR-Cas9 vectors from various sources were adapted for use in *C. botulinum* and tested for utility in mutagenesis. The commercially available genome editing vector pNICKclos2.0 was a gift from Sheng Yang (Addgene plasmid # 73228). A sequence targeting the gene of interest was purchased as gBlock DNA from IDT (Table 3). All DNA was stored at -20°C. Oligonucleotide primers were designed to amplify homology arms flanking the target sequence. Upstream and downstream segments of 1 kb each were generated by PCR using the primer pairs described in Table 3. A novel PstI restriction site was introduced between the homology arms to allow for detection of successful genome editing.

Cells were prepared for electroporation by subculturing three times in standard TPGY. Following the third passage, 500 µl of the culture was inoculated into 50 ml TPGY + 1% glycine and incubated at 37°C. Once the culture reached an OD_{600} of 0.5, it was centrifuged at 6000 x *g* for 7 minutes. The pellet was washed once in a buffer of 270 mM sucrose, 1 mM $MgCl_2$, and 7 mM Na_2HPO_4 (pH 7.4). The cells were then suspended in 500 µl of a 10% PEG 8000 solution. A series of cuvettes containing 2 µg plasmid DNA were used as vessels for the electroporation; 70 µl of cell suspension was added to each cuvette, mixed gently, and incubated at ambient temperature for 10 minutes. Using a BTX ECM 830 machine (Harvard Apparatus, Holliston, MA), samples were electroporated at 1800 V for 125 µs. Following the pulse, each was immediately transferred to 10 ml pre-warmed TPGY with 20 mM $MgCl_2$ and incubated at 34°C for 5 h. Outgrowth cultures were plated on selective TYG agar and incubated anaerobically at 37°C to recover colonies.

Table 3. Strains, plasmids, and oligonucleotides used in this work. All oligonucleotides were purchased from Integrated DNA Technologies (Coralville, IA). Restriction sites are underlined; CRISPR guide sequences are bolded.

Name	Description	Source or Reference
C. botulinum Hall A-*hyper*	Group I *C. botulinum*, BoNT/A1 producer	(42)
E. coli CA434	Plasmid donor strain	(29, 30)
pMTL80000 series	*E. coli-Clostridium* shuttle vectors with modular promoters, antibiotic resistance genes, and replication sequences	(29)
pMTL9361	Clostridial inducible expression vector, Em^R	(52); gift of N.P. Minton
pJP042	RecT1 inducible expression vector for ssDNA recombineering, Em^R	(53)
pNICKclos2.0	All-in-one genome editing vector containing Cas9 nickase, Em^R	(54)
pJIR751	*E. coli-Clostridium* shuttle vector, Em^R	(55)
recT$_{Cb}$ fwd	ATGGCAAATACAAAAGC	
recT$_{Cb}$ rev	TTATTCTTGTTTAGCTTC	
pNICKclos2.0-*bont*-gBlock	CCTAGGTATAATACTAGT**AATTATAAAGATCCTGTAAA**GTTTT AGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCA ACTTGAAAAAGTGGCACCGAGTCGGTGCTTTTTTGCGGCCGC	
*bont*HAleft fwd	GCGGCCGCAATCAAAGTATAAGTTTTTCTAATG	
*bont*HAleft rev	ACCCTGCAGAAATTGTTTATTAACAAATGGC	
*bont*HAright fwd	CTGCAGTGGTGTTGATATTGCTTATATAAAAATTC	
*bont*HAright rev	CTCGAGAATCTCTGTTAACATTTTGTATAACTTATC	

Results

A pilot study was performed to evaluate the potential for oligonucleotide-mediated genetic engineering, or recombineering, to introduce unmarked mutations to *C. botulinum*. Recombineering in a given strain requires a functional *recT* gene under the control of an inducible promoter and the efficient transformation of ssDNA into the strain. Two inducible systems were examined: the IPTG-induced pMTL9361 vector (52) and the sakacin P-based expression system kindly provided by the van Pijkeren laboratory on pJP042 (Table 3). pJP042 carries the *L. reuteri recT1* gene, which was successful in recombineering various *Lactobacillus* species (53). As the functionality of this gene was unknown in *Clostridium*, we searched publicly available *C. botulinum* genomes for *recT* homologs and identified a match in the Hall strain (NC_009698), locus tag CLC_2421. This gene was successfully amplified by PCR from a genomic DNA template using the *recT*$_{Cb}$ primer pair (Figure 4).

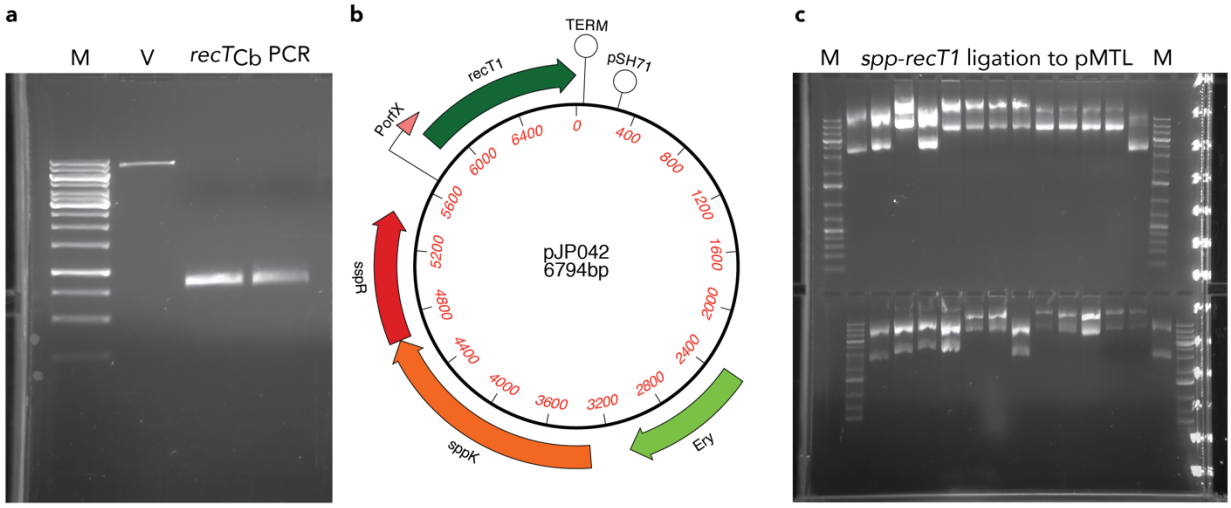

Figure 4. *C. botulinum* contains a *recT* homologue. (a) PCR was conducted on a genomic DNA template using the *recT*$_{Cb}$ primers given in Table 3. In parallel, (b) the *spp-recT1* fragment from pJP042 was (c) ligated to the modular vectors of the pMTL80000 series to allow replication in *C. botulinum*. M, 1 kb Gene Ruler; V, pMTL9361 vector control. Plasmid map constructed using MacVector software.

As an alternative strategy, we cloned the *sppKR-recT1* fragment from pJP042 into the pMTL modular vectors, ensuring that the vectors would stably replicate in *C. botulinum* and

eliminating the need to electroporate the plasmid into the host strain (Figure 4). This plasmid was introduced by conjugation into Hall A-*hyper* and induction of the *L. reuteri recT1* gene was attempted with the proper inducer peptide (MAGNSSNFIHKIKQIFTHR; Peptide2.0) (53). The culture was prepared for electroporation of the mutagenic oligonucleotides, but no transformant cells were obtained. As this was a very rudimentary experiment performed without proper reporters or optimization parameters, we refrain from making any conclusions about the feasibility of recombineering in *C. botulinum*. We pursued other strategies more zealously as a consequence of our focus on plasmid genes; it was unclear at the time whether recombineering oligonucleotides could be incorporated into extrachromosomal DNA, as the mechanism of BoNT plasmid replication in *C. botulinum* is not well characterized. If the plasmid genetics become evident, or if future research focuses on *C. botulinum* chromosomal loci instead, there are many opportunities to investigate recombineering as a viable method in the species and optimize its use, from oligonucleotide design to growth conditions (56).

Assorted attempts to implement CRISPR-Cas9 genome editing in *C. botulinum* were also performed. An all-in-one CRISPR-Cas9 nickase vector, available for purchase on Addgene, has been used for genome editing in other *Clostridium* species (54). This plasmid, pNICKclos2.0, incorporates a guide sequence targeting the gene of interest and homology arms that flank the editing site. A guide sequence was designed to target *bont/a* in *C. botulinum* Hall A-*hyper* and synthesized as a gBlock from IDT (Table 3). The homology arms were set as 1 kb regions up- and downstream of the target site and were amplified from genomic DNA by PCR (Figure 5). These reactions relied on primers that introduce a novel PstI restriction site at the junction between the two arms (Table 3). The novel site allows for populations to be screened by PCR and restriction digest for cells that have repaired CRISPR-Cas9 cuts with the provided homology template. Unedited cells will produce PCR amplicons without the PstI site.

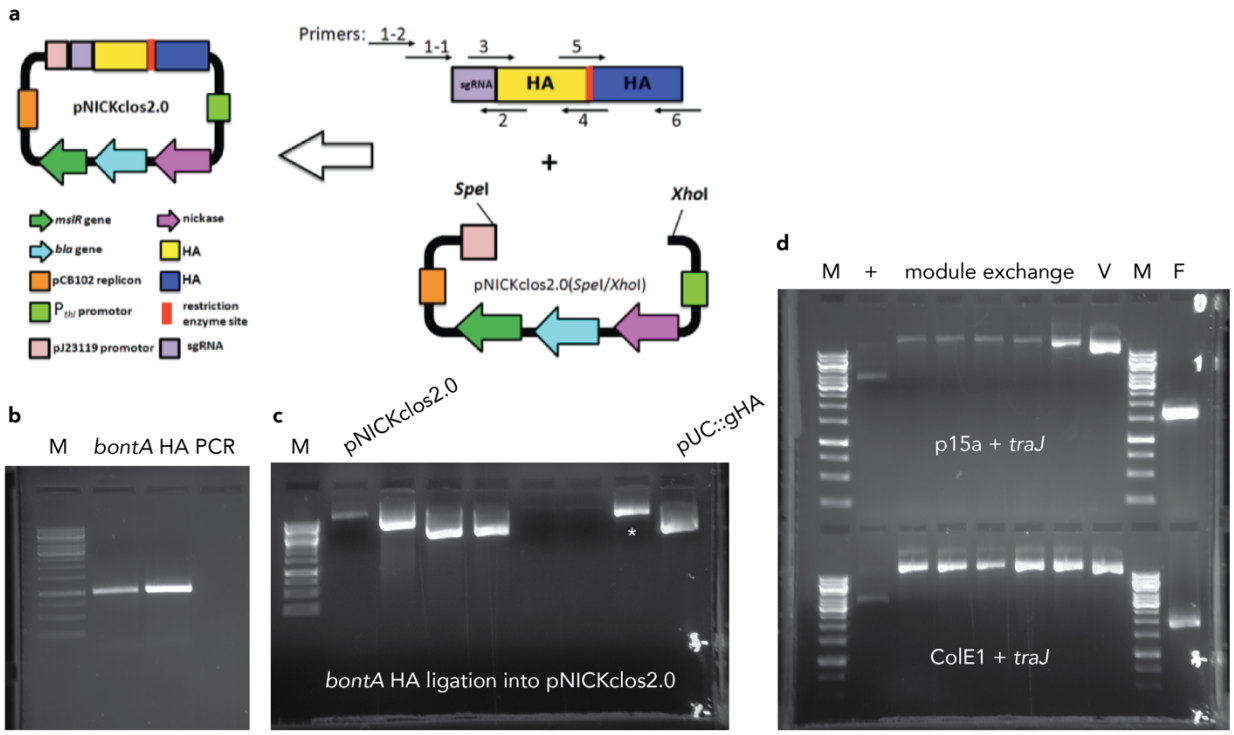

Figure 5. Cloning strategy for CRISPR-Cas9n vector assembly. (a) Addgene plasmid #73228, also known as pNICKclos2.0, was provided by Sheng Yang. The vector diagram is reproduced from Li et al. (54). (b) Homology arms (HA) 1 kb upstream and downstream of the *bontA* target site were amplified by PCR from genomic DNA. (c) They were then ligated together with the aid of a novel PstI restriction site and fused to a gBlock DNA segment providing the Cas9 guide sequence. These three fragments were maintained in a pUC cloning vector (pUC::gHA), then subcloned by restriction digest into the SpeI-XhoI sites of pNICKclos2.0. The starred construct was confirmed to have the proper configuration with additional restriction digests. (d) Later, the Gram-negative replication sequence of pNICKclos2.0 (not shown) was replaced with the p15a + *traJ* or ColE1 + *traJ* modules from the pMTL80000 series. M, 1 kb Gene Ruler; +, Gibson assembly positive control; V, pNICKclos2.0 vector; F, pMTL Gram-negative replication module amplified by PCR.

The commercial pNICKclos2.0 targets the *xylR* gene of *Clostridium beijerinckii*. No such gene is annotated in the genome of *C. botulinum* Hall A-*hyper*, and the guide sequence gives no significant homology to *C. botulinum* in a BLAST query (57), suggesting that the *xylR* targeting construct could be used as a negative control in this strain. Both pNICKclos2.0-*xylR* and its derivative pNICKclos2.0-*bontA*, which we assembled by PCR and restriction digest cloning, were introduced into *C. botulinum* Hall A-*hyper* by electroporation. No Em^R colonies were recovered in either case. In order to determine whether the pNICKclos2.0 constructs were

simply inefficient in electroporation or whether they were lethal to *C. botulinum*, we performed a two-fold troubleshooting approach. First, we tested the electroporation efficiency of the pNICKclos2.0-*bontA* construct in a panel of clostridia, including one that lacks the *bont/a* gene. Second, we swapped the replication sequence of pNICKclos2.0-*bontA* for the pMTL modules that are permissible to conjugation from *E. coli* donors (Figure 5). pNICKclos2.0-*bontA* was successfully recovered (as evidenced by Em^R colonies) only in the *C. botulinum* strain missing the *bont/a* toxin cluster. These data suggest that the cuts introduced by the Cas9 nickase may be lethal to *C. botulinum* under our experimental conditions, and perhaps that the pNICKclos2.0-*xylR* targeting construct also has off-target lethal effects in *C. botulinum* Hall A-*hyper*. It is also possible that the nontoxigenic *C. botulinum* strain, LNT01, has a greater tolerance for foreign DNA than the others tested. Further studies are necessary to interrogate these effects. Shortly after our efforts to introduce pNICKclos2.0 into *C. botulinum*, other groups reported that a new clostridial CRISPR-Cas system was forthcoming, although this has not yet been made commercially available (N.P. Minton, personal communication).

Discussion

Controlled expression is crucial to the development of clostridial genetic tools

Genetic tools in *C. botulinum* are greatly underdeveloped when compared to other bacterial species, even other clostridia. The two strategies investigated here, recombineering and CRISPR-Cas9 genome editing, both enable rapid mutagenesis of clostridia without the insertion of markers or scars. Improved methods would open up numerous and far-reaching avenues of research into the genetics and physiology of *C. botulinum*. The generation of unmarked mutants could create new models for investigating BoNT production and regulation, processes that are always of special interest in the organism.

In this work, the implementation of genetic tools was hampered by poorly controlled gene expression in *C. botulinum*: the proper method of inducing *recT* expression for recombineering applications was not determined in *C. botulinum* Hall A-*hyper*, nor was constitutive expression of the Cas9 nickase successful in directing genome editing in the organism. Tighter transcriptional control of either of these heterologous genes could accelerate the development of genome editing in *C. botulinum*. In addition to the IPTG-inducible pMTL9361 vector (Table 3), which can be leaky, future experiments might benefit from screening other promoters that are induced by xylose (58) or by anhydrotetracycline (59), two systems that have found purchase in *C. acetobutylicum* and related species. The necessities of a vast promoter library and a robust reporter system in clostridia were highlighted in a recent review of synthetic biology in the genus (60).

Both recombineering and CRISPR-Cas could also be optimized in *C. botulinum* by mining clostridial genomes for homologues to the effector proteins. *recT* genes tend to exhibit species specificity, although the genetic distance between organisms is not the sole determinant of recombineering efficiency (61). A *recT* gene from *C. perfringens*, for example, was used to

develop a method of recombineering *C. acetobutylicum* (62). It is not clear whether the *recT* homologue identified in *C. botulinum* Hall A-*hyper* in this study is the optimal recombinase for the organism; there are a number of other BLAST hits for *recT* across the clostridia that might be viable candidates (Figure 6).

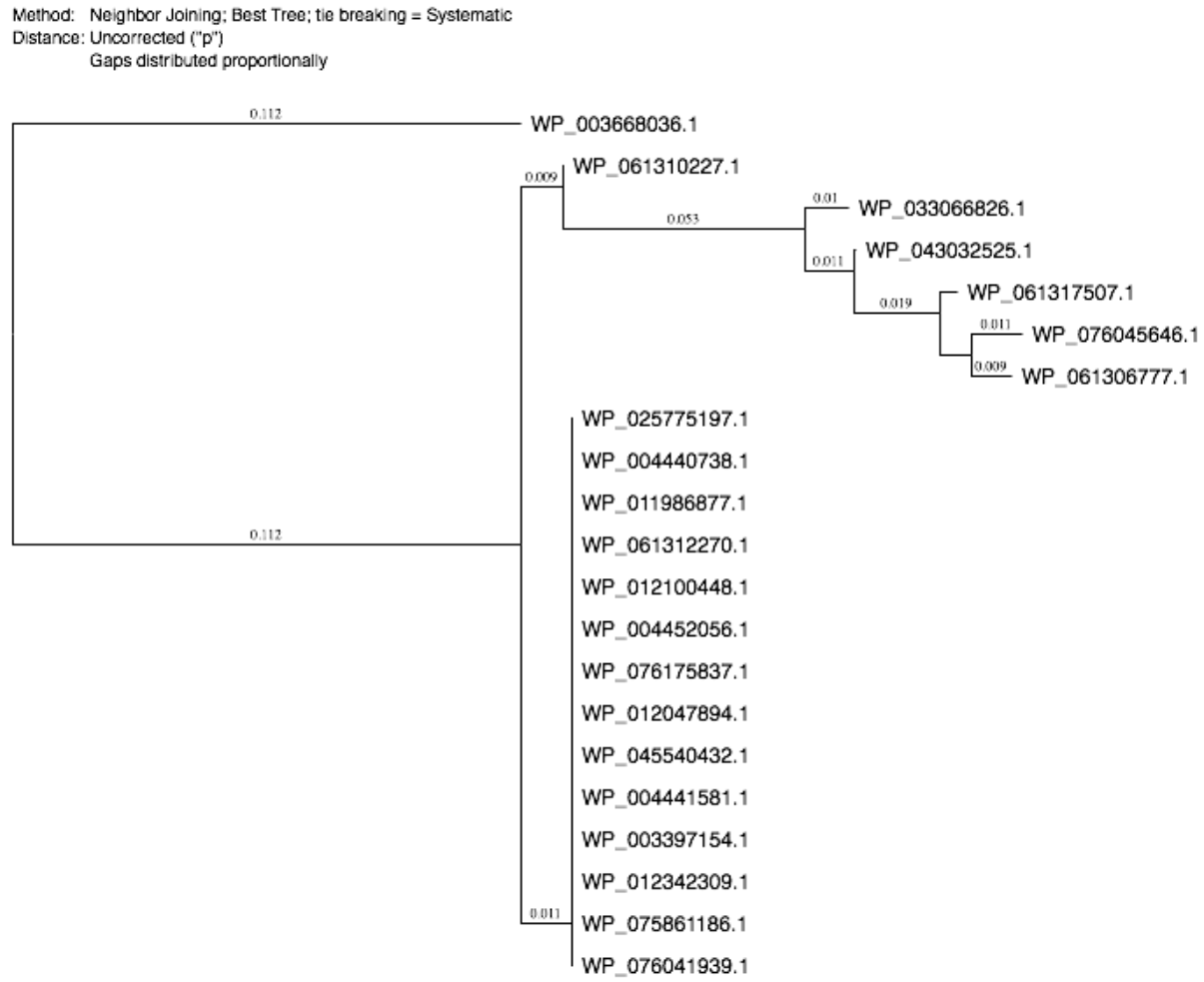

Figure 6. *C. botulinum* is rich in RecT homologues. The *C. botulinum* Hall A *recT* gene described in the text served as the query sequence in a BLASTx search of all non-redundant *C. botulinum* protein sequences (63). The top twenty proteins producing significant sequence alignments to the query were aligned to each other in MacVector software using the ClustalW algorithm (64). A *Lactobacillus reuteri* RecT sequence was included in the alignment as an outgroup, and the resulting tree was rooted at this node (top branch, accession number WP_003668036).

Endogenous Type I-B CRISPR-Cas systems have also been exploited in the clostridia with greater editing efficiencies than those achieved by traditional heterologous CRISPR-Cas9

machinery (65, 66). A wide variety of CRISPR arrays and *cas* genes have been bioinformatically identified in *C. botulinum* genomes in several physiological Groups, perhaps serving as an untapped source of genetic tools with varied specificities and enzymatic modes of action (67). Recent reports from other clostridial genetics laboratories indicate that CRISPR-Cas9 genome editing can be implemented in *C. botulinum*, but that it has concerning off-target effects, emphasizing the necessity of obtaining whole genome sequences before characterizing a purported mutant (N.P. Minton, M. Lindström, personal communications).

Roadblocks to genetic engineering of C. botulinum

While *C. botulinum* and related species harbor great potential for the development of medically and industrially relevant molecules, they present a number of genetic challenges that must be overcome. The first barrier is the introduction of foreign DNA into the cell. In *C. botulinum*, electroporation is a fairly inefficient process requiring large quantities of plasmid DNA (68). Conjugation from *E. coli* is more routine, but necessitates the use of vectors with Gram-negative replicons and suitable donor strains (29). The Hall A-*hyper* strain transformed in this work is an ideal target for molecular manipulations, owing to its lack of surface layer proteins (69) and a possible deletion in the mismatch repair system (N.P. Minton, personal communication). Other strains of *C. botulinum*, however, can be problematic—and as the strains bearing this species designation are loosely related on the genomic scale, they are likely to require individual optimization. In silico analysis of the endogenous CRISPR arrays in *C. botulinum* strains bears out this assumption, indicating that strains from various Groups are diversely equipped to defend against invasion by foreign plasmids and bacteriophage (67).

Assuming that DNA is successfully introduced into the target cell, one must then contend with the inefficiency of homologous recombination in *C. botulinum*. Double crossover events

are rare in clostridia and must be paired to another technique for ease of use (60, 70, 71). In the system known as allelic coupled exchange (ACE), homologous recombination is coupled to a counter-selection method, such as fluoroacetic acid sensitivity via the *pyrE* gene (72). ACE is not a one-step method, but it is reliable and useful in many clostridia, and works synergistically with other mutagenic strategies including ClosTron and CRISPR (71). While we initially pursued recombineering as a means of avoiding homologous recombination in *C. botulinum*, it seems that the route to implementing an ACE-based system would be equally feasible. This is particularly true in light of the fact that the ACE vectors are already engineered for use in *Clostridium*.

The diversity of *C. botulinum* strains is often perceived as a nuisance. In some cases, however, as in the easily transformed Hall A-*hyper*, genomic polymorphisms are an asset. When the experimental design allows, future research might benefit from an expanded pool of target strains. Careful choice (or a dedicated screen) of possible recipient strains may reveal genetic biases that enable more rapid editing. Even within a single-strain population, there are likely polymorphisms that can facilitate editing. The transformation efficiency of the solvent-producing species *C. pasteurianum* was improved by several orders of magnitude when "hypertransformable variants" were isolated from standard electroporations (73). A similar strategy might be fruitful in *C. botulinum*, and in fact it is possible that the colonies recovered in our assays have obtained mutations that make them hypertransformable. Until a deeper sequence analysis is undertaken, the genetic basis of these transformants will remain unknown.

Despite the substantial challenges of genetic engineering in *C. botulinum*, tools for manipulating the organism continue to improve. Electroporation and conjugation protocols, for instance, are typically aided by heat shock of the culture (74). Efforts to overcome the restriction modification barrier are useful in various clostridia, relying on a set of *E. coli* plasmid donor

strains with diverse methylase activities (71). Once these fundamental techniques are optimized for target strains of *C. botulinum*, more sophisticated methodologies such as recombineering and CRISPR-Cas9 genome editing are sure to follow.

Works Cited

1. Schiavo G, Benfenati F, Poulain B, Rossetto O, Polverino de Laureto P, DasGupta BR, Montecucco C. Tetanus and botulinum-B neurotoxins block neurotransmitter release by proteolytic cleavage of synaptobrevin. Nature. 1992;359(6398):832-5. Epub 1992/10/29. doi: 10.1038/359832a0. PubMed PMID: 1331807.
2. Schiavo G, Rossetto O, Catsicas S, Polverino de Laureto P, DasGupta BR, Benfenati F, Montecucco C. Identification of the nerve terminal targets of botulinum neurotoxin serotypes A, D, and E. J Biol Chem. 1993;268(32):23784-7. Epub 1993/11/15. PubMed PMID: 8226912.
3. Schiavo G, Shone CC, Rossetto O, Alexander FCG, Montecucco C. Botulinum Neurotoxin Serotype-F Is a Zinc Endopeptidase Specific for Vamp/Synaptobrevin. Journal of Biological Chemistry. 1993;268(16):11516-9. PubMed PMID: WOS:A1993LF28400012.
4. Schiavo G, Malizio C, Trimble WS, Delaureto PP, Milan G, Sugiyama H, Johnson EA, Montecucco C. Botulinum-G Neurotoxin Cleaves Vamp/Synaptobrevin at a Single Ala-Ala Peptide-Bond. Journal of Biological Chemistry. 1994;269(32):20213-6. PubMed PMID: WOS:A1994PB31700002.
5. Schiavo G, Shone CC, Bennett MK, Scheller RH, Montecucco C. Botulinum Neurotoxin Type-C Cleaves a Single Lys-Ala Bond within the Carboxyl-Terminal Region of Syntaxins. Journal of Biological Chemistry. 1995;270(18):10566-70. doi: DOI 10.1074/jbc.270.18.10566. PubMed PMID: WOS:A1995QW60100033.
6. Simpson LL. Molecular pharmacology of botulinum toxin and tetanus toxin. Annu Rev Pharmacol Toxicol. 1986;26:427-53. Epub 1986/01/01. doi: 10.1146/annurev.pa.26.040186.002235. PubMed PMID: 3521461.
7. Montecucco C, Schiavo G. Structure and function of tetanus and botulinum neurotoxins. Q Rev Biophys. 1995;28(4):423-72. Epub 1995/11/01. doi: 10.1111/j.1365-2958.1994.tb00396.x. PubMed PMID: 8771234.
8. Montal M. Botulinum neurotoxin: a marvel of protein design. Annual review of biochemistry. 2010;79:591-617. Epub 2010/03/18. doi: 10.1146/annurev.biochem.051908.125345. PubMed PMID: 20233039.
9. Peck MW, Smith TJ, Anniballi F, Austin JW, Bano L, Bradshaw M, Cuervo P, Cheng LW, Derman Y, Dorner BG, Fisher A, Hill KK, Kalb SR, Korkeala H, Lindstrom M, Lista F, Luquez C, Mazuet C, Pirazzini M, Popoff MR, Rossetto O, Rummel A, Sesardic D, Singh BR, Stringer SC. Historical Perspectives and Guidelines for Botulinum Neurotoxin Subtype Nomenclature. Toxins. 2017;9(1). Epub 2017/01/21. doi: 10.3390/toxins9010038. PubMed PMID: 28106761; PMCID: PMC5308270.
10. Smith TJ, Lou J, Geren IN, Forsyth CM, Tsai R, Laporte SL, Tepp WH, Bradshaw M, Johnson EA, Smith LA, Marks JD. Sequence variation within botulinum neurotoxin serotypes impacts antibody binding and neutralization. Infect Immun. 2005;73(9):5450-7. Epub 2005/08/23. doi: 10.1128/IAI.73.9.5450-5457.2005. PubMed PMID: 16113261; PMCID: PMC1231122.
11. Keller JE, Neale EA, Oyler G, Adler M. Persistence of botulinum neurotoxin action in cultured spinal cord cells. FEBS Lett. 1999;456(1):137-42. Epub 1999/08/19. PubMed PMID: 10452545.
12. Whitemarsh RC, Tepp WH, Johnson EA, Pellett S. Persistence of botulinum neurotoxin A subtypes 1-5 in primary rat spinal cord cells. PLoS One. 2014;9(2):e90252. Epub 2014/03/04. doi: 10.1371/journal.pone.0090252. PubMed PMID: 24587301; PMCID: PMC3937374.

13. Adler M, Keller JE, Sheridan RE, Deshpande SS. Persistence of botulinum neurotoxin A demonstrated by sequential administration of serotypes A and E in rat EDL muscle. Toxicon. 2001;39(2-3):233-43. Epub 2000/09/09. PubMed PMID: 10978741.
14. Pier CL, Chen C, Tepp WH, Lin G, Janda KD, Barbieri JT, Pellett S, Johnson EA. Botulinum neurotoxin subtype A2 enters neuronal cells faster than subtype A1. FEBS Lett. 2011;585(1):199-206. Epub 2010/12/04. doi: 10.1016/j.febslet.2010.11.045. PubMed PMID: 21126520; PMCID: PMC3041152.
15. Whitemarsh RC, Tepp WH, Bradshaw M, Lin G, Pier CL, Scherf JM, Johnson EA, Pellett S. Characterization of botulinum neurotoxin A subtypes 1 through 5 by investigation of activities in mice, in neuronal cell cultures, and in vitro. Infect Immun. 2013;81(10):3894-902. Epub 2013/08/07. doi: 10.1128/IAI.00536-13. PubMed PMID: 23918782; PMCID: PMC3811745.
16. Moriishi K, Koura M, Abe N, Fujii N, Fujinaga Y, Inoue K, Ogumad K. Mosaic structures of neurotoxins produced from *Clostridium botulinum* types C and D organisms. Biochim Biophys Acta. 1996;1307(2):123-6. Epub 1996/06/07. PubMed PMID: 8679691.
17. Dover N, Barash JR, Hill KK, Xie G, Arnon SS. Molecular characterization of a novel botulinum neurotoxin type H gene. J Infect Dis. 2014;209(2):192-202. Epub 2013/10/10. doi: 10.1093/infdis/jit450. PubMed PMID: 24106295.
18. Pickett A, Perrow K. Towards new uses of botulinum toxin as a novel therapeutic tool. Toxins. 2011;3(1):63-81. Epub 2011/11/10. doi: 10.3390/toxins3010063. PubMed PMID: 22069690; PMCID: PMC3210455.
19. Bradshaw M, Tepp WH, Whitemarsh RC, Pellett S, Johnson EA. Holotoxin Activity of Botulinum Neurotoxin Subtype A4 Originating from a Nontoxigenic *Clostridium botulinum* Expression System. Appl Environ Microbiol. 2014;80(23):7415-22. Epub 2014/09/23. doi: 10.1128/AEM.01795-14. PubMed PMID: 25239905; PMCID: PMC4249187.
20. Moreira GM, Cunha CE, Salvarani FM, Goncalves LA, Pires PS, Conceicao FR, Lobato FC. Production of recombinant botulism antigens: a review of expression systems. Anaerobe. 2014;28:130-6. Epub 2014/06/17. doi: 10.1016/j.anaerobe.2014.06.003. PubMed PMID: 24930432.
21. Band PA, Blais S, Neubert TA, Cardozo TJ, Ichtchenko K. Recombinant derivatives of botulinum neurotoxin A engineered for trafficking studies and neuronal delivery. Protein Expr Purif. 2010;71(1):62-73. Epub 2010/01/05. doi: 10.1016/j.pep.2009.12.013. PubMed PMID: 20045734; PMCID: PMC2830362.
22. Dong J, Thompson AA, Fan Y, Lou J, Conrad F, Ho M, Pires-Alves M, Wilson BA, Stevens RC, Marks JD. A single-domain llama antibody potently inhibits the enzymatic activity of botulinum neurotoxin by binding to the non-catalytic alpha-exosite binding region. J Mol Biol. 2010;397(4):1106-18. Epub 2010/02/09. doi: 10.1016/j.jmb.2010.01.070. PubMed PMID: 20138889; PMCID: PMC2903050.
23. Vazquez-Cintron EJ, Vakulenko M, Band PA, Stanker LH, Johnson EA, Ichtchenko K. Atoxic derivative of botulinum neurotoxin A as a prototype molecular vehicle for targeted delivery to the neuronal cytoplasm. PLoS One. 2014;9(1):e85517. Epub 2014/01/28. doi: 10.1371/journal.pone.0085517. PubMed PMID: 24465585; PMCID: PMC3899041.
24. Keller JE. Recovery from botulinum neurotoxin poisoning in vivo. Neuroscience. 2006;139(2):629-37. Epub 2006/02/24. doi: 10.1016/j.neuroscience.2005.12.029. PubMed PMID: 16490322.
25. Hanahan D, Jessee J, Bloom FR. Plasmid transformation of *Escherichia coli* and other bacteria. Methods Enzymol. 1991;204:63-113. Epub 1991/01/01. PubMed PMID: 1943786.

26. Whitmer ME, Johnson EA. Development of Improved Defined Media for *Clostridium botulinum* Serotype-A, Serotype-B, and Serotype-E. Applied and Environmental Microbiology. 1988;54(3):753-9. Epub 1988/03/01. PubMed PMID: WOS:A1988M313600022; PMCID: PMC202536.
27. Hill KK, Smith TJ. Genetic diversity within *Clostridium botulinum* serotypes, botulinum neurotoxin gene clusters and toxin subtypes. Curr Top Microbiol Immunol. 2013;364:1-20. Epub 2012/12/15. doi: 10.1007/978-3-642-33570-9_1. PubMed PMID: 23239346.
28. Bradshaw M, Marshall KM, Heap JT, Tepp WH, Minton NP, Johnson EA. Construction of a nontoxigenic *Clostridium botulinum* strain for food challenge studies. Appl Environ Microbiol. 2010;76(2):387-93. Epub 2009/11/26. doi: 10.1128/AEM.02005-09. PubMed PMID: 19933346; PMCID: PMC2805223.
29. Heap JT, Pennington OJ, Cartman ST, Minton NP. A modular system for *Clostridium* shuttle plasmids. J Microbiol Methods. 2009;78(1):79-85. Epub 2009/05/19. doi: 10.1016/j.mimet.2009.05.004. PubMed PMID: 19445976.
30. Purdy D, O'Keeffe TAT, Elmore M, Herbert M, McLeod A, Bokori-Brown M, Ostrowski A, Minton NP. Conjugative transfer of clostridial shuttle vectors from *Escherichia coli* to *Clostridium difficile* through circumvention of the restriction barrier. Molecular Microbiology. 2002;46(2):439-52. doi: DOI 10.1046/j.1365-2958.2002.03134.x. PubMed PMID: WOS:000178861100012.
31. Pellett S, Tepp WH, Stanker LH, Band PA, Johnson EA, Ichtchenko K. Neuronal targeting, internalization, and biological activity of a recombinant atoxic derivative of botulinum neurotoxin A. Biochem Biophys Res Commun. 2011;405(4):673-7. Epub 2011/02/03. doi: 10.1016/j.bbrc.2011.01.093. PubMed PMID: 21284937; PMCID: PMC3046291.
32. Pellett S, Bradshaw M, Tepp WH, Pier CL, Whitemarsh RCM, Chen C, Barbieri JT, Johnson EA. The Light Chain Defines the Duration of Action of Botulinum Toxin Serotype A Subtypes. MBio. 2018;9(2). Epub 2018/03/29. doi: 10.1128/mBio.00089-18. PubMed PMID: 29588398; PMCID: PMC5874905.
33. Hill KK, Xie G, Foley BT, Smith TJ. Genetic diversity within the botulinum neurotoxin-producing bacteria and their neurotoxins. Toxicon. 2015;107(Pt A):2-8. Epub 2015/09/15. doi: 10.1016/j.toxicon.2015.09.011. PubMed PMID: 26368006.
34. Lin G, Tepp WH, Bradshaw M, Fredrick CM, Johnson EA. Immunoprecipitation of native botulinum neurotoxin complexes from *Clostridium botulinum* subtype A strains. Appl Environ Microbiol. 2015;81(2):481-91. Epub 2014/11/02. doi: 10.1128/AEM.02817-14. PubMed PMID: 25362065; PMCID: PMC4277567.
35. Nugent M, Yusef YR, Meng J, Wang J, Dolly JO. A SNAP-25 cleaving chimera of botulinum neurotoxin /A and /E prevents TNFalpha-induced elevation of the activities of native TRP channels on early postnatal rat dorsal root ganglion neurons. Neuropharmacology. 2018;138:257-66. Epub 2018/06/16. doi: 10.1016/j.neuropharm.2018.06.016. PubMed PMID: 29906413.
36. Barash JR, Arnon SS. A novel strain of *Clostridium botulinum* that produces type B and type H botulinum toxins. J Infect Dis. 2014;209(2):183-91. Epub 2013/10/10. doi: 10.1093/infdis/jit449. PubMed PMID: 24106296.
37. Pellett S, Tepp WH, Bradshaw M, Kalb SR, Dykes JK, Lin G, Nawrocki EM, Pier CL, Barr JR, Maslanka SE, Johnson EA. Purification and Characterization of Botulinum Neurotoxin FA from a Genetically Modified *Clostridium botulinum* Strain. mSphere. 2016;1(1). Epub 2016/06/16. doi: 10.1128/mSphere.00100-15. PubMed PMID: 27303710; PMCID: PMC4863619.

38. Marshall KM, Bradshaw M, Johnson EA. Conjugative botulinum neurotoxin-encoding plasmids in *Clostridium botulinum*. PLoS One. 2010;5(6):e11087. Epub 2010/06/17. doi: 10.1371/journal.pone.0011087. PubMed PMID: 20552020; PMCID: PMC2884020.
39. Perutka J, Wang W, Goerlitz D, Lambowitz AM. Use of computer-designed group II introns to disrupt *Escherichia coli* DExH/D-box protein and DNA helicase genes. J Mol Biol. 2004;336(2):421-39. Epub 2004/02/06. PubMed PMID: 14757055.
40. Heap JT, Pennington OJ, Cartman ST, Carter GP, Minton NP. The ClosTron: a universal gene knock-out system for the genus *Clostridium*. J Microbiol Methods. 2007;70(3):452-64. Epub 2007/07/31. doi: 10.1016/j.mimet.2007.05.021. PubMed PMID: 17658189.
41. Nawrocki EM, Bradshaw M, Johnson EA. Botulinum neurotoxin-encoding plasmids can be conjugatively transferred to diverse clostridial strains. Scientific reports. 2018;8(1):3100. Epub 2018/02/17. doi: 10.1038/s41598-018-21342-9. PubMed PMID: 29449580; PMCID: PMC5814558.
42. Schantz EJ, Johnson EA. Properties and Use of Botulinum Toxin and Other Microbial Neurotoxins in Medicine. Microbiological Reviews. 1992;56(1):80-99. PubMed PMID: WOS:A1992HH37500005.
43. Smith TJ, Hill KK, Foley BT, Detter JC, Munk AC, Bruce DC, Doggett NA, Smith LA, Marks JD, Xie G, Brettin TS. Analysis of the neurotoxin complex genes in *Clostridium botulinum* A1-A4 and B1 strains: BoNT/A3, /Ba4 and /B1 clusters are located within plasmids. PLoS One. 2007;2(12):e1271. Epub 2007/12/07. doi: 10.1371/journal.pone.0001271. PubMed PMID: 18060065; PMCID: PMC2092393.
44. Heap JT, Kuehne SA, Ehsaan M, Cartman ST, Cooksley CM, Scott JC, Minton NP. The ClosTron: Mutagenesis in *Clostridium* refined and streamlined. J Microbiol Methods. 2010;80(1):49-55. Epub 2009/11/07. doi: 10.1016/j.mimet.2009.10.018. PubMed PMID: 19891996.
45. Maslanka SE, Luquez C, Dykes JK, Tepp WH, Pier CL, Pellett S, Raphael BH, Kalb SR, Barr JR, Rao A, Johnson EA. A Novel Botulinum Neurotoxin, Previously Reported as Serotype H, Has a Hybrid-Like Structure With Regions of Similarity to the Structures of Serotypes A and F and Is Neutralized With Serotype A Antitoxin. J Infect Dis. 2016;213(3):379-85. Epub 2015/06/13. doi: 10.1093/infdis/jiv327. PubMed PMID: 26068781; PMCID: PMC4704661.
46. Pellett S, Tepp WH, Lin G, Johnson EA. Substrate cleavage and duration of action of botulinum neurotoxin type FA ("H, HA"). Toxicon. 2018;147:38-46. Epub 2017/12/24. doi: 10.1016/j.toxicon.2017.12.048. PubMed PMID: 29273248; PMCID: PMC5911199.
47. Iyer LM, Koonin EV, Aravind L. Classification and evolutionary history of the single-strand annealing proteins, RecT, Redbeta, ERF and RAD52. BMC Genomics. 2002;3(1):8. Epub 2002/03/27. PubMed PMID: 11914131; PMCID: PMC101383.
48. Yu DG, Ellis HM, Lee EC, Jenkins NA, Copeland NG, Court DL. An efficient recombination system for chromosome engineering in *Escherichia coli*. Proceedings of the National Academy of Sciences of the United States of America. 2000;97(11):5978-83. Epub 2000/05/17. doi: DOI 10.1073/pnas.100127597. PubMed PMID: WOS:000087318700056; PMCID: PMC18544.
49. Datta S, Costantino N, Zhou X, Court DL. Identification and analysis of recombineering functions from Gram-negative and Gram-positive bacteria and their phages. Proc Natl Acad Sci U S A. 2008;105(5):1626-31. Epub 2008/01/31. doi: 10.1073/pnas.0709089105. PubMed PMID: 18230724; PMCID: PMC2234195.
50. Van Pijkeren JP, Neoh KM, Sirias D, Findley AS, Britton RA. Exploring optimization parameters to increase ssDNA recombineering in *Lactococcus lactis* and *Lactobacillus reuteri*.

Bioengineered. 2012;3(4):209-17. Epub 2012/07/04. doi: 10.4161/bioe.21049. PubMed PMID: 22750793; PMCID: PMC3476877.
51. Jiang WY, Bikard D, Cox D, Zhang F, Marraffini LA. RNA-guided editing of bacterial genomes using CRISPR-Cas systems. Nature Biotechnology. 2013;31(3):233-9. doi: 10.1038/nbt.2508. PubMed PMID: WOS:000316439500018.
52. Pier CL, Tepp WH, Bradshaw M, Johnson EA, Barbieri JT, Baldwin MR. Recombinant holotoxoid vaccine against botulism. Infect Immun. 2008;76(1):437-42. Epub 2007/10/31. doi: 10.1128/IAI.00843-07. PubMed PMID: 17967862; PMCID: PMC2223665.
53. van Pijkeren JP, Britton RA. High efficiency recombineering in lactic acid bacteria. Nucleic Acids Res. 2012;40(10):e76. Epub 2012/02/14. doi: 10.1093/nar/gks147. PubMed PMID: 22328729; PMCID: PMC3378904.
54. Li Q, Chen J, Minton NP, Zhang Y, Wen Z, Liu J, Yang H, Zeng Z, Ren X, Yang J, Gu Y, Jiang W, Jiang Y, Yang S. CRISPR-based genome editing and expression control systems in *Clostridium acetobutylicum* and *Clostridium beijerinckii*. Biotechnology journal. 2016;11(7):961-72. Epub 2016/05/24. doi: 10.1002/biot.201600053. PubMed PMID: 27213844.
55. Bannam TL, Rood JI. *Clostridium perfringens-Escherichia coli* shuttle vectors that carry single antibiotic resistance determinants. Plasmid. 1993;29(3):233-5. Epub 1993/05/01. doi: 10.1006/plas.1993.1025. PubMed PMID: 8356117.
56. Sawitzke JA, Costantino N, Li XT, Thomason LC, Bubunenko M, Court C, Court DL. Probing cellular processes with oligo-mediated recombination and using the knowledge gained to optimize recombineering. J Mol Biol. 2011;407(1):45-59. Epub 2011/01/25. doi: 10.1016/j.jmb.2011.01.030. PubMed PMID: 21256136; PMCID: PMC3046259.
57. Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. Basic local alignment search tool. J Mol Biol. 1990;215(3):403-10. Epub 1990/10/05. doi: 10.1016/S0022-2836(05)80360-2. PubMed PMID: 2231712.
58. Girbal L, Mortier-Barriere I, Raynaud F, Rouanet C, Croux C, Soucaille P. Development of a sensitive gene expression reporter system and an inducible promoter-repressor system for *Clostridium acetobutylicum*. Appl Environ Microbiol. 2003;69(8):4985-8. Epub 2003/08/07. PubMed PMID: 12902297; PMCID: PMC169153.
59. Dong H, Tao W, Zhang Y, Li Y. Development of an anhydrotetracycline-inducible gene expression system for solvent-producing *Clostridium acetobutylicum*: A useful tool for strain engineering. Metab Eng. 2012;14(1):59-67. Epub 2011/11/08. doi: 10.1016/j.ymben.2011.10.004. PubMed PMID: 22056607.
60. Joseph RC, Kim NM, Sandoval NR. Recent Developments of the Synthetic Biology Toolkit for *Clostridium*. Front Microbiol. 2018;9:154. Epub 2018/02/28. doi: 10.3389/fmicb.2018.00154. PubMed PMID: 29483900; PMCID: PMC5816073.
61. van Pijkeren JP, Britton RA. Precision genome engineering in lactic acid bacteria. Microb Cell Fact. 2014;13 Suppl 1(Suppl 1):S10. Epub 2014/09/05. doi: 10.1186/1475-2859-13-S1-S10. PubMed PMID: 25185700; PMCID: PMC4155826.
62. Dong H, Tao W, Gong F, Li Y, Zhang Y. A functional *recT* gene for recombineering of *Clostridium*. J Biotechnol. 2014;173:65-7. Epub 2014/01/05. doi: 10.1016/j.jbiotec.2013.12.011. PubMed PMID: 24384234.
63. Altschul SF, Madden TL, Schaffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. 1997;25(17):3389-402. Epub 1997/09/01. PubMed PMID: 9254694; PMCID: PMC146917.
64. Thompson JD, Higgins DG, Gibson TJ. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap

penalties and weight matrix choice. Nucleic Acids Res. 1994;22(22):4673-80. Epub 1994/11/11. PubMed PMID: 7984417; PMCID: PMC308517.
65. Pyne ME, Bruder MR, Moo-Young M, Chung DA, Chou CP. Harnessing heterologous and endogenous CRISPR-Cas machineries for efficient markerless genome editing in *Clostridium*. Scientific reports. 2016;6:25666. Epub 2016/05/10. doi: 10.1038/srep25666. PubMed PMID: 27157668; PMCID: PMC4860712.
66. Zhang J, Zong W, Hong W, Zhang ZT, Wang Y. Exploiting endogenous CRISPR-Cas system for multiplex genome editing in *Clostridium tyrobutyricum* and engineer the strain for high-level butanol production. Metab Eng. 2018;47:49-59. Epub 2018/03/14. doi: 10.1016/j.ymben.2018.03.007. PubMed PMID: 29530750.
67. Negahdaripour M, Nezafat N, Hajighahramani N, Rahmatabadi SS, Ghasemi Y. Investigating CRISPR-Cas systems in *Clostridium botulinum* via bioinformatics tools. Infect Genet Evol. 2017;54:355-73. Epub 2017/07/08. doi: 10.1016/j.meegid.2017.06.027. PubMed PMID: 28684374.
68. Zhou YT, Johnson EA. Genetic Transformation of *Clostridium botulinum* Hall A by Electroporation. Biotechnology Letters. 1993;15(2):121-6. doi: Doi 10.1007/Bf00133010. PubMed PMID: WOS:A1993KP30900004.
69. Ng V, Lin WJ. Comparison of assembled *Clostridium botulinum* A1 genomes revealed their evolutionary relationship. Genomics. 2014;103(1):94-106. Epub 2013/12/27. doi: 10.1016/j.ygeno.2013.12.003. PubMed PMID: 24369123; PMCID: PMC3959226.
70. Al-Hinai MA, Fast AG, Papoutsakis ET. Novel system for efficient isolation of *Clostridium* double-crossover allelic exchange mutants enabling markerless chromosomal gene deletions and DNA integration. Appl Environ Microbiol. 2012;78(22):8112-21. Epub 2012/09/18. doi: 10.1128/AEM.02214-12. PubMed PMID: 22983967; PMCID: PMC3485963.
71. Minton NP, Ehsaan M, Humphreys CM, Little GT, Baker J, Henstra AM, Liew F, Kelly ML, Sheng L, Schwarz K, Zhang Y. A roadmap for gene system development in *Clostridium*. Anaerobe. 2016;41:104-12. Epub 2016/05/29. doi: 10.1016/j.anaerobe.2016.05.011. PubMed PMID: 27234263; PMCID: PMC5058259.
72. Heap JT, Ehsaan M, Cooksley CM, Ng YK, Cartman ST, Winzer K, Minton NP. Integration of DNA into bacterial chromosomes from plasmids without a counter-selection marker. Nucleic Acids Res. 2012;40(8):e59. Epub 2012/01/20. doi: 10.1093/nar/gkr1321. PubMed PMID: 22259038; PMCID: PMC3333862.
73. Grosse-Honebrink A, Schwarz KM, Wang H, Minton NP, Zhang Y. Improving gene transfer in *Clostridium pasteurianum* through the isolation of rare hypertransformable variants. Anaerobe. 2017;48:203-5. Epub 2017/09/14. doi: 10.1016/j.anaerobe.2017.09.001. PubMed PMID: 28899647; PMCID: PMC5734229.
74. Kirk JA, Fagan RP. Heat shock increases conjugation efficiency in *Clostridium difficile*. Anaerobe. 2016;42:1-5. Epub 2016/11/05. doi: 10.1016/j.anaerobe.2016.06.009. PubMed PMID: 27377776; PMCID: PMC5154368.

Conclusions, Perspectives, and Future Directions

The research presented herein has broadened our understanding of BoNT-encoding plasmids in *C. botulinum*, beginning with an exploration of a plasmid's host range (Chapter One) and narrowing to an essential conjugation gene on said plasmid (Chapter Two). As there are numerous other BoNT-encoding plasmids carrying uncharacterized clostridial conjugation genes, there are ample avenues for future research that would build on this thesis. The primary challenge will be approaching these problems in a higher-throughput fashion. Unfortunately, this work was unable to implement a more rapid system of genome editing in *C. botulinum* that would expand the possibilities for clostridial research (Chapter Three). Our lab and others will continue to pursue genetic tools in *C. botulinum*. Some applications of these tools, and their utility in investigating conjugation in this species, are discussed in these concluding remarks.

Analysis of pCLJ donor and transconjugant genotypes

While there are likely a number of physical and environmental determinants of conjugation efficiency in *C. botulinum*, a screen for inducing conditions would be arduous under the current methodology. Genomic and transcriptional analyses may provide alternate paths to understanding pCLJ transfer in *C. botulinum* strain 657Ba. On the genomic front, one could envision a sequencing project that compared a subset of the pCLJ transconjugants generated in Chapters One and Two. Two to four isolates of each transconjugant have been maintained in glycerol stocks at -80°C. Whole genome analysis might show whether these strains carry mutations at certain loci that are associated with the uptake of pCLJ. If a suitable mutagenesis technique emerges, one could also create a mutant library in a single donor background and screen this library for increases and decreases in transfer frequency. The Em^R pCLJ donor and

the Tm^R recipient described elsewhere in this work would be ideal mating partners for this assay, which could be performed in 96-well plates or by pinning on agar.

On the transcriptomic front, strains in mating pairs could be sampled at various time points to give insight into the dynamics of plasmid transfer. In the past, our laboratory has been hesitant to undertake an RNA sequencing project exploring the conjugation process. The low transfer frequency of pCLJ would indicate that perhaps one donor cell in a million is actively conjugating, and it would be difficult to mine meaningful transcriptomic data from such a rare event. Now that CLJ_0213 is known to play a role in pCLJ transfer, however, a quantitative PCR approach targeting this and other genes in the conjugation region might be useful. qPCR has been used to investigate transcript levels of plasmid regulatory genes in an *E. coli* population where 10% of cells are estimated to be competent for transfer of the pCTX-M3 plasmid (1). A fuller sequence-based characterization method, either by DNA or RNA, would certainly help guide future research on conjugation in *C. botulinum*.

Characterization of the pCLJ cell wall hydrolase

In addition to deep sequencing, it would be wise to pursue a few molecular targets that are likely to be involved in pCLJ transfer. Several of the target genes that we insertionally inactivated using the Em^R ClosTron in Chapter Two seemed to reduce the frequency of pCLJ transfer but not abolish it, suggesting that there may be functional redundancy in the conjugation system. One component that may not be redundant is the cell wall hydrolase. The most likely hydrolase candidate on pCLJ, bearing the locus tag CLJ_0235, is predicted to have an N-terminal signal sequence, a transmembrane helix, and an active site that binds and cleaves peptidoglycan linkages. Homologues of this gene product are found in other *C. botulinum* genomes, but no significant matches are found elsewhere in *C. botulinum* 657Ba. We therefore think it likely that

inactivation of CLJ_0235 with an Em^R ClosTron would be prohibitive to pCLJ transfer. If this is the case, there are mutagenic experiments and biochemical assays that could demonstrate the activity of the enzymatic domain in CLJ_0235. A framework for the characterization of the peptidoglycan hydrolase can be found in the work of Bantwal et al., who demonstrated a significant reduction in pCW3 transfer frequency in a *C. perfringens tcpG* mutant (2). The *C. perfringens* mutant could be complemented in *trans*, and purified TcpG had peptidoglycan hydrolyzing activity (2). This group later showed that TcpG associates with TcpC, the major assembly factor for the *C. perfringens* pCW3 conjugation apparatus (3). The strength of the peptidoglycan hydrolase mutant phenotype in *C. perfringens* suggests that *C. botulinum* conjugation research might find a rewarding target in CLJ_0235.

Identification of the pCLJ oriT

Turning to stricter plasmid biology, this project would benefit from a classification of key mobilization components. In Gram-positive bacteria, conjugation is typified by two transfer mechanisms (4). The first, a double-stranded DNA transfer reminiscent of chromosomal segregation, is thus far exclusive to the *Actinomycetales* and will not be discussed here. The second, a single-stranded DNA transfer through type IV secretion system (T4SS) components, is a more likely model for *C. botulinum*, as it occurs in other clostridia and is suggested by genome annotations on Group I *C. botulinum* plasmids. Binding at the *oriT* and nicking the *nic* site are the first steps in conjugation using a T4SS. Identification of the relaxase responsible is therefore of paramount importance and may represent a possible drug target in *C. botulinum* and related clostridia. Genetically, the identification of an *oriT* and relaxase would enable proper classification of the plasmids with respect to their molecular mechanism of transfer and broaden our understanding of conjugation in *C. botulinum*.

As *oriT*s are typically found nearby the genes responsible for conjugative plasmid transfer, one could initially restrict the search to the "conjugation region" on pCLJ. In the related *C. perfringens*, the *oriT* of pCW3 is found in the intergenic region between two *tcp* genes (5). A blastn search (6) querying this 391 bp intergenic region against *C. botulinum* 657Ba detected a match within plasmid genes CLJ_0206 and CLJ_0207. The 150 bp minimal fragment of pCW3's *oriT* is 56% identical to the respective region on plasmid pCLJ, suggesting this region as a candidate *oriT* (Figure 1).

```
  1 AAGGAACTTTACAGGGAACTTTAAAAATTTAAATTGATATAAAAGTTCCCTGTATTAGTA  60
  1 AAACTATTTTATGCGTTGCTTTTAATTTTTTACTATATATGATTGCATTATCTATTGATG  60
    **   * ****   *   **** **  *** * *  **** *  *     * ****  *

 61 TAAGTATTTTAAAGGTATATATCATTATTAGTTCCTTATCGTATTATAGAGTATATATTA 120
 61 TAAATTTTAATTTGTAATCCAATATATTTTCCCCCTTGTT-TATGATAGAAT-TATCTTC 118
    *** * **     *  **  *  **  **    **** *  *** ***** * *** **

121 TATATATAATATATACA-----TATAATGTATTGG 150
119 TTTAATCAATCTATATAATCTTTATAATGACTTGG 153
    * **   *** **** *     *******  ****
```

Figure 1. The minimal *oriT* fragment from *C. perfringens* plasmid pCW3 aligns to the conjugation region of pCLJ. The genome of *C. botulinum* strain 657Ba was probed with pCW3's *oriT* as described in the text (5). A matching sequence in the pCLJ conjugation region (bottom row) was then aligned to the minimal pCW3 *oriT* fragment (top row) using ClustalW (7). Identical nucleotides are marked with an asterisk.

Using some combination of ClosTron markers and CRISPR-Cas9 editing, pCLJ could be tagged at precise loci around the length of the plasmid. Interrupted matings could then be used to define the transfer frequencies of the various markers (8). As in chromosomal mapping experiments, the plasmid markers closest to the *oriT* are transferred first and would be found with highest frequency in recipients. A library of nonconjugative vectors could simultaneously be prepared to contain pCLJ fragments. Those carrying the pCLJ *oriT* would be mobilized from an unmarked *C. botulinum* 657Ba strain by its resident pCLJ conjugation machinery. Site-

directed mutagenesis of the *oriT* region could then be undertaken to determine the nucleotides essential for nicking.

Identification of the pCLJ relaxase

The relaxase responsible for conjugal transfer is typically encoded near the *oriT* (4). Extending the comparison to *C. perfringens*, future work in *C. botulinum* could attempt to identify and validate the pCLJ relaxase by analogy to TcpM (5). TcpM is encoded directly upstream of the *C. perfringens* pCW3 *oriT* and specifically binds and nicks the *oriT* sequence (5). TcpM does not have a clear homologue on Group I *C. botulinum* plasmids. However, relaxases from various plasmid incompatibility groups share common motifs, as they must fulfill common functions, e.g. DNA binding and cleavage and docking with other proteins as part of the relaxosome complex (9). TraI, the canonical relaxase of the *E. coli* F plasmid (10), contains three functional domains: a relaxase, a helicase, and a C-terminal protein-protein interaction domain (11).

Using a PSI-BLAST search (12) of *C. botulinum* 657Ba proteins for matches to TraI, we identified candidate gene products with similar functions. CLJ_0205 is a hypothetical protein with predicted AAA ATPase activity and tandem tyrosine residues near its N-terminus, a conserved motif that is found in the active site of many relaxases (9, 13). CLJ_0205 itself does not have multifunctional domain annotations, but it is found adjacent to a putative helicase (CLJ_0204). It is possible that the two work in tandem to nick and unwind DNA for conjugal transfer. They could be expressed and purified for use in biochemical techniques such as EMSAs or plasmid relaxation assays. Alternatively, as the technology allows, a library of pCLJ mutants could be generated by transposon insertions or CRISPR-Cas9 edits. As described

above, a mutant library would be incredibly useful in defining the conjugative apparatus on pCLJ.

Insights into C. botulinum *and its neurotoxins*

Although dissecting the mechanisms of conjugation in *C. botulinum* plasmids will require further efforts, the work presented here is an important starting point. In particular, the exploration of pCLJ's host range and mobility could help shed light on the relationship between *C. botulinum* and BoNTs. Clostridial strains and the neurotoxins they encode are bound together by evolution, but the ties that bind them—in other words, the selective advantage that *bont* genes provide—are not immediately evident. The plasmid-borne toxins offer an intriguing avenue of research into this question. As demonstrated in Chapter One, novel bacterial strains can be converted to toxigenicity by the acquisition of a BoNT-encoding plasmid. The persistence of plasmid replicons is somewhat of a paradox, however (14). Why do cells maintain plasmid-borne genes, rather than carrying them chromosomally or simply losing them to segregation? The likeliest explanation is that plasmids give bacteria flexibility (15). The presence of extrachromosomal genes in the gene pool can allow a population to adapt quickly to changing environments (16). When these genes are transmissible, as in the case of conjugative plasmids, the adaptation can quickly spread. Perhaps the *bont* genes, or some as-yet-unidentified accessory functions on pCLJ, are advantageous not in laboratory conditions but rather in unique natural situations. Having extended the host range of pCLJ, and thus the environmental range of the strains that can produce BoNTs, we can now investigate a wider set of circumstances under which these toxins may exist.

A variety of experiments might be designed to probe the relationship between the bacteria and their plasmid-borne toxins. Competition assays comparing the head-to-head fitness

of two strains could reveal new physiological nuances. It would be interesting to test, for example, whether a strain with a plasmid-borne *bont* gene is more or less fit than a strain with a chromosomal toxin, and whether fitness advantages are dependent on culture conditions. Plasmid genes might contribute to survival in nutrient-limiting environments, or they might allow increased virulence or colonization in intestinal models of botulism. These experiments would be ideal uses for the second ClosTron marker described in Chapter Two.

Looking further ahead, the research presented here could provide a template for assaying plasmid stability and replication. Inactivating a gene of interest with the ClosTron has been valuable for following the transfer of pCLJ in mating pairs, but the same technique would also allow for dedicated study of plasmid genes within a single strain. Certain loci on pCLJ might be essential for partitioning the plasmid, in which case their inactivation would lead to errors in plasmid copy number. Higher-resolution genetic and molecular analyses, aided by the markers and novel host strains introduced in this work, could illuminate the mechanics of pCLJ's segregation and replication. The BoNT-encoding plasmids in Group I *C. botulinum* are altogether poorly characterized, and any such insights into their biology would represent a step forward for the field.

Works Cited

1. Dmowski M, Golebiewski M, Kern-Zdanowicz I. Characteristics of the Conjugative Transfer System of the IncM Plasmid pCTX-M3 and Identification of Its Putative Regulators. J Bacteriol. 2018;200(18). Epub 2018/07/11. doi: 10.1128/JB.00234-18. PubMed PMID: 29986941; PMCID: PMC6112013.
2. Bantwal R, Bannam TL, Porter CJ, Quinsey NS, Lyras D, Adams V, Rood JI. The peptidoglycan hydrolase TcpG is required for efficient conjugative transfer of pCW3 in *Clostridium perfringens*. Plasmid. 2012;67(2):139-47. Epub 2012/01/17. doi: 10.1016/j.plasmid.2011.12.016. PubMed PMID: 22244927.
3. Porter CJ, Bantwal R, Bannam TL, Rosado CJ, Pearce MC, Adams V, Lyras D, Whisstock JC, Rood JI. The conjugation protein TcpC from *Clostridium perfringens* is structurally related to the type IV secretion system protein VirB8 from Gram-negative bacteria. Mol Microbiol. 2012;83(2):275-88. Epub 2011/12/14. doi: 10.1111/j.1365-2958.2011.07930.x. PubMed PMID: 22150951.
4. Goessweiner-Mohr N, Arends K, Keller W, Grohmann E. Conjugation in Gram-Positive Bacteria. Microbiol Spectr. 2014;2(4):PLAS-0004-2013. Epub 2015/06/25. doi: 10.1128/microbiolspec.PLAS-0004-2013. PubMed PMID: 26104193.
5. Wisniewski JA, Traore DA, Bannam TL, Lyras D, Whisstock JC, Rood JI. TcpM: a novel relaxase that mediates transfer of large conjugative plasmids from *Clostridium perfringens*. Molecular Microbiology. 2016;99(5):884-96. Epub 2015/11/13. doi: 10.1111/mmi.13270. PubMed PMID: WOS:000371241600007.
6. Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. Basic local alignment search tool. J Mol Biol. 1990;215(3):403-10. Epub 1990/10/05. doi: 10.1016/S0022-2836(05)80360-2. PubMed PMID: 2231712.
7. Thompson JD, Higgins DG, Gibson TJ. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res. 1994;22(22):4673-80. Epub 1994/11/11. PubMed PMID: 7984417; PMCID: PMC308517.
8. Chen J, Pappas DL, Galli DM. Mapping of the nick site on conjugative plasmid pVT745 by interrupted mating. Plasmid. 2010;63(3):136-42. Epub 2010/02/11. doi: 10.1016/j.plasmid.2010.02.001. PubMed PMID: 20144651; PMCID: PMC2840195.
9. Zechner EL, Lang S, Schildbach JF. Assembly and mechanisms of bacterial type IV secretion machines. Philosophical transactions of the Royal Society of London Series B, Biological sciences. 2012;367(1592):1073-87. Epub 2012/03/14. doi: 10.1098/rstb.2011.0207. PubMed PMID: 22411979; PMCID: PMC3297438.
10. Traxler BA, Minkley EG, Jr. Evidence that DNA helicase I and *oriT* site-specific nicking are both functions of the F TraI protein. J Mol Biol. 1988;204(1):205-9. Epub 1988/11/05. PubMed PMID: 2851049.
11. Matson SW, Ragonese H. The F-plasmid TraI protein contains three functional domains required for conjugative DNA strand transfer. J Bacteriol. 2005;187(2):697-706. Epub 2005/01/05. doi: 10.1128/JB.187.2.697-706.2005. PubMed PMID: 15629940; PMCID: PMC543555.
12. Altschul SF, Koonin EV. Iterated profile searches with PSI-BLAST--a tool for discovery in protein databases. Trends in biochemical sciences. 1998;23(11):444-7. Epub 1998/12/16. PubMed PMID: 9852764.

13. Garcillan-Barcia MP, Francia MV, de la Cruz F. The diversity of conjugative relaxases and its application in plasmid classification. FEMS Microbiol Rev. 2009;33(3):657-87. Epub 2009/04/28. doi: 10.1111/j.1574-6976.2009.00168.x. PubMed PMID: 19396961.
14. Carroll AC, Wong A. Plasmid persistence: costs, benefits, and the plasmid paradox. Can J Microbiol. 2018;64(5):293-304. Epub 2018/03/22. doi: 10.1139/cjm-2017-0609. PubMed PMID: 29562144.
15. Eberhard WG. Why do bacterial plasmids carry some genes and not others? Plasmid. 1989;21(3):167-74. Epub 1989/05/01. PubMed PMID: 2675150.
16. San Millan A, MacLean RC. Fitness Costs of Plasmids: a Limit to Plasmid Transmission. Microbiol Spectr. 2017;5(5). Epub 2017/09/26. doi: 10.1128/microbiolspec.MTBP-0016-2017. PubMed PMID: 28944751.

Appendix: Additional mating experiments exploring the transfer of pCLJ

Abstract

The bivalent BoNT plasmid pCLJ was previously shown to be mobilized from its host strain to a variety of other clostridia. These experiments, detailed in Chapter One of this work, relied on the generation of a lysine auxotrophy in the donor strain and an Em^R ClosTron insertion in the plasmid's *bont/b* gene. Here, taking advantage of the representative Tm^R recipient strain discussed in Chapter Two, we have once again probed the host range of pCLJ in an attempt to better define its conjugal transfer. Following its initial transfer from the lys^- donor to an unmarked recipient background, pCLJ can be subsequently be transferred from this transconjugant strain to a new recipient. This observation lends credence to the hypothesis that pCLJ is a conjugative plasmid. Still, there may be chromosomal factors that aid in or promote the transfer of pCLJ. We examined whether the presence of *Enterococcus* cultures or spent supernatants in the mating mixtures of two *C. botulinum* strains modulated the transfer frequency of pCLJ. No significant increase in transfer frequency was observed, suggesting that the given *C. botulinum* conjugation reaction is not induced by enterococcal pheromones. A broader survey of culture conditions is likely to uncover factors that stimulate the transfer of pCLJ, and a high-throughput mating assay could be adapted for this purpose.

Introduction

Plasmids are abundant in bacterial genomes, encoding a variety of physiological assets like virulence factors and antibiotic resistance genes (1). Gram-positive bacteria, including the Firmicutes, are no exception (2-4). When cells grow in community, their intraspecies and interspecies interactions can promote horizontal gene transfer (HGT) (5). If toxin plasmids are horizontally transferred between bacteria, the resulting strains can have novel properties including increased virulence and pathogenicity (6). While HGT encompasses a variety of DNA transfer processes, conjugation is a special case: for a plasmid to be deemed conjugative, it must encode its own mating pair formation complex, a membrane-associated channel through which DNA can be transferred (7). This is in addition to the origin of transfer, relaxase, and type IV coupling protein that make a plasmid mobilizable (7).

The transfer of conjugative elements between bacteria is induced by a variety of stimuli. Focusing strictly on Firmicutes, in the *Enterococci*, soluble factors known as sex pheromones are released by recipient cells and taken up by donor cells, where they derepress transcription of essential plasmid transfer genes (8, 9). In *Bacillus* species, the mobility of ICE*Bs*1 elements can be similarly activated by large concentrations of recipient cells or by the SOS response to DNA damage (10). These and other prokaryotic phyla also induce conjugation in response to environmental signals, such as temperature or nutrient availability (11).

Conjugative elements themselves can also stimulate plasmid transfer between strains. As discussed in Chapter One of this work, Tn*916* is a conjugative transposon that is capable of mobilizing nonconjugative plasmids, so long as they encode an appropriate *mob* locus and origin of transfer (12-14). A bioinformatic survey of Mob proteins across all bacterial genomes suggests that a large percentage of plasmids are mobilizable (7). All told, the process of conjugative plasmid transfer is governed by complex regulatory networks and interspecies

interactions. Thus, while characterizing conjugation in a pairwise mating is informative, incorporating additional strains carrying additional conjugative elements may reveal new contributions to a target plasmid's transfer.

In *C. botulinum*, plasmids are found in many sequenced genomes, including all Group III strains (15, 16). More intriguingly, plasmids are found in several Group I bivalent strains isolated from cases of infant botulism (17-20). These include the *C. botulinum* 657Ba strain that forms the basis of this work (17). In addition, although the bivalent infant botulism strain that is the source of BoNT/FA (also known as BoNT/H; see Chapter Three) does not carry a toxin plasmid, the hybrid *bont* gene is flanked on the chromosome by a pCLJ-like region (21). The prevalence of plasmids and plasmid-like sequences in all of these strains is suggestive of recombination events, possibly driven by conjugation in the infectious infant botulism environment. With this in mind, we attempted to determine whether (1) *C. botulinum* strains that obtained pCLJ in conjugal matings could transfer the plasmid to new hosts and (2) whether coculture with a representative intestinal bacterium could stimulate pCLJ transfer.

Methods

Biosafety and biosecurity

Clostridium botulinum and BoNTs are classified as Tier 1 Category A Select Agents, the highest security group of biological agents. The Johnson laboratory and personnel are registered with the Federal Select Agent Program for research involving BoNTs and BoNT-producing strains of clostridia. The research program, procedures, documentation, security, and facilities are closely monitored by the University of Wisconsin-Madison Biosecurity Task Force, University of Wisconsin-Madison Office of Biological Safety, University of Wisconsin Select Agent Program, and the Centers for Disease Control and Prevention. All personnel continually undergo suitability assessments and rigorous and biosafety training, including biosafety level 3 (BSL3) or BSL2 and select agent practices, before participating in laboratory studies involving BoNTs and neurotoxigenic *C. botulinum*. All plasmid transfer experiments were conducted using plasmid pCLJ on which the major *bont* gene had been genetically inactivated by ClosTron (22). The second plasmid-borne *bont* gene produces the minor toxin BoNT/A4, which has an LD_{50} greater than 100 ng/kg (23, 24). As the toxicity is above this threshold, creating new strains of bacteria that express BoNT/A4 is not designated as a restricted experiment/major action according to the Federal Select Agent Regulations and the *NIH Guidelines*.

Bacterial strains and culture conditions

Clostridial strains were maintained at 37°C in TPGY broth (50 g/l trypticase peptone, 5 g/l Bacto peptone, 4 g/l dextrose, 20 g/l yeast extract, 1 g/l cysteine-HCl, pH 7.4), and on TYG agar (30 g/l Bacto tryptone, 20 g/l yeast extract, 1 g/l sodium thioglycolate). *Enterococcus faecalis* strains were grown aerobically on brain heart infusion (BHI) media at 37°C. Frozen stocks consisted of TPGY cultures supplemented with 20% glycerol and stored at -80°C.

Erythromycin was used at 50 μg/ml, thiamphenicol at 15 μg/ml, and tetracycline at 10 μg/ml where appropriate. All chemicals and media components were purchased from Becton Dickinson Microbiology Systems (Sparks, MD) and Sigma-Aldrich (St. Louis, MO) unless otherwise noted.

Clostridial cultures were grown under anaerobic conditions. Glass culture tubes were flushed with nitrogen gas and sealed with butyl rubber stoppers (Bellco Glass, Vineland, NJ) before sterilizing. All culture manipulations were performed in an anaerobic chamber (Forma Anaerobic System, Marietta, OH) with an initial gas mixture of 80% N_2, 10% CO_2, and 10% H_2. Resazurin was added to solid media at 2 μg/ml, and agar plates were prereduced by overnight incubation in the anaerobic chamber.

Plasmid transfer

Donor and recipient strains were revived from frozen stock, subcultured twice, and grown to $OD_{600} \approx 0.8$. In order to enumerate donors and recipients, 100 μl of each culture was spotted individually on TYG agar. To assess plasmid transfer, equal volumes (100 μl) of donor and recipient were spotted together on TYG agar. In experiments with *E. faecium*, 100 μl culture fluid or 100 μl filtered supernatant was spotted on mating plates together with the donor and recipient cultures. All mating plates were allowed to incubate uninverted at 37°C for 16 h. Bacterial growth was then washed with 3 ml 1 × PBS, and the suspensions were serially diluted in 1 × PBS. Dilutions of donor and recipient controls were plated on TYG with Em and on TYG with Tm, respectively, to determine CFU/ml. Aliquots of donor and recipient controls were also spread on inhibitory plates to test for spontaneous mutation. Mating mixtures were diluted and plated on TYG supplemented with Em and Tm to enumerate transconjugants. Transfer

frequency was calculated by dividing transconjugant CFU/ml by the larger of the donor or recipient CFU/ml.

Results

pCLJ transconjugants can transfer the plasmid to new recipients

The development of a Tm^R ClosTron marker, described in Chapter Two of this work, presented a number of new possibilities for tracking the transfer of pCLJ. First, in order to support the hypothesis that pCLJ encodes the genes responsible for its own transfer, we performed mating experiments in which a lys^+Em^R transconjugant served as the donor to a Tm^R recipient. Three pCLJ transconjugant strains were chosen as donors: *C. sporogenes* (pCLJ), *C. botulinum* LNT01 (pCLJ), and Hall A-*hyper* (pCLJ). *C. botulinum* Hall A-*hyper bont/a*::*catP* served as recipient in each mating pair. Of the three, the intrastrain Hall A-*hyper* mating pair was the only that generated true, quantifiable transconjugants (Table 1). No colonies were recovered on erythromycin + thiamphenicol selective plates when *C. botulinum* LNT01 (pCLJ) served as donor. The *C. sporogenes* (pCLJ) donor strain alone grew in the presence of thiamphenicol, indicating that this marker is unsuitable for selection against the given donor background.

Table 1. pCLJ can be mobilized from Hall A-*hyper* transconjugants. The pCLJ plasmid in each donor strain below is tagged with an Em^R ClosTron insertion in the *bont/b* gene and was acquired via mating with *C. botulinum* 657Ba lys^- (see Chapter One). Transfer frequency was calculated by dividing transconjugant CFU/ml by the larger of the donor or recipient CFU/ml.

Donor	Recipient	Transfer Frequency
C. sporogenes (pCLJ)	Hall A-*hyper* Tm^R	Undetermined, Em^RTm^R colonies TNTC
C. botulinum LNT01 (pCLJ)	Hall A-*hyper* Tm^R	N/A, Em^RTm^R colonies TFTC
C. botulinum Hall A-*hyper* (pCLJ)	Hall A-*hyper* Tm^R	9.6×10^{-12}

Coculture with Enterococcus *does not stimulate pCLJ transfer*

Turning our attention next to the stimulation of pCLJ conjugation by extracellular factors, we performed experiments in which the donor and recipient strain were supplemented with either whole culture or filtered culture supernatant from a mid-exponential phase culture of *E. faecalis*. *E. faecalis* is the source of the Tn*916* transposon described elsewhere in this work and was

maintained in the presence of tetracycline (25). Neither culture nor supernatant of *E. faecalis* induced pCLJ conjugation, as the plasmid's frequency of transfer from donor to recipient *C. botulinum* was nearly identical in all scenarios (Table 2).

Table 2. pCLJ transfer is not significantly stimulated by the addition of *E. faecalis* culture fluid or supernatant. *C. botulinum* 657Ba, carrying the pCLJ *bont/b*::*ermB* plasmid, was the donor in all matings. *C. botulinum* Hall A-*hyper bont/a*::*catP* was the recipient. Strains were grown to an approximate OD_{600} of 0.8 and mated on TYG agar plates as previously described. Transfer frequency was calculated by dividing the transconjugant CFU/ml by the larger of the donor or the recipient CFU/ml. The only transconjugant selection was for the BoNT-encoding plasmid in the target *C. botulinum* recipient (i.e. pCLJ transfer into *E. faecalis* was not monitored).

E. faecalis component in mating mixture	Mean Transfer Frequency
None	2.4×10^{-12}
Whole culture	1.3×10^{-12}
Filtered supernatant	8.2×10^{-13}

Discussion

Building on our experiments demonstrating pCLJ transfer in biparental mating pairs, we here indicate that the plasmid can be mobilized from a transconjugant to a new recipient strain (Table 1). This is suggestive of pCLJ's conjugative ability, although the fact that only one transconjugant strain was a permissive donor is certainly worthy of additional study. The apparent inability of LNT01 (pCLJ) to mobilize its plasmid to Hall A-*hyper bont/a*::*catP* may be due to missing accessory factors or poor compatibility with the recipient. Conjugative transfer is typically susceptible to multilevel regulation by both the internal environment (the plasmid's interaction with its host chromosome) and the external environment (the surrounding culture conditions, including other inhabitants of the niche) (26). Internally, the LNT01 strain has a large region deleted from its chromosome, including the *bont* toxin cluster locus (27). Externally, it is thought to produce a bacteriocin that is inhibitory or microbicidal to other *C. botulinum* cells (22). The signaling pathways and/or cell-cell contacts necessary for pCLJ conjugation may therefore be defective or rare in LNT01 matings.

In contrast, pCLJ was transferred with relative ease in intrastrain conjugation assays (Table 1). All mating assays performed in this work have taken place on a rich medium of solid agar, allowing high cell densities to accumulate overnight. Might the transfer of pCLJ be induced by a quorum sensing system? A number of species use this strategy. Conjugation is an energy-intensive process, and donor cells are wise to delay it until the population of potential recipient cells is sufficiently large (28). *C. botulinum* contains multiple *agr* quorum sensing loci homologous to those first characterized in *S. aureus* (29). The *agr* system is involved in sporulation and neurotoxin pathways in *C. botulinum*, but beyond that little is known about its regulon in the species (29, 30).

Finally, despite efforts to stimulate pCLJ transfer with the addition of a third party to the mating mixtures, no significant increase in transconjugants was observed (Table 2). As discussed above, the signaling cascade that leads to induction of pCLJ conjugation in *C. botulinum* is unclear, and the changes in transcription that occur when *C. botulinum* strains are grown in coculture have not been investigated. A recent study of *E. faecalis* conjugation *in vivo* shed light on several factors that might influence plasmid transfer in the environment (31). First, a small reduction in transfer efficiency was observed when a competing microbial community was present in the intestinal tract, as compared to transfer in a germfree mouse model (31). Second, transfer of the pCF10 plasmid in *E. faecalis* demonstrated a preference for particular segments of the intestinal tract. At just five hours post-inoculation, the upper and middle intestinal sites were conducive to plasmid transfer in nearly all mice sampled, while the lower intestine supported plasmid transfer in only two of six mice (31). These data indicate that conjugation can proceed rapidly under proper conditions, but that the local environment is a major determinant of transfer efficiency. Similar stratification of horizontal gene transfer is seen in the biofilm environment (32).

It is clear that transfer of pCLJ has been a matter of trial and error thus far. While the single *E. faecalis* strain tested did not seem to induce *C. botulinum* conjugation under our culture conditions, there may be merit in screening a larger panel of strains, perhaps multiplexing them as a better mimic of the intestinal microbiome. Current mating assays on agar plates are poorly scalable, but preliminary experiments demonstrate that the transfer of pCLJ *bont/b*::*ermB* to Hall A-*hyper bont/a*::*catP* can be detected in liquid media in 96-well plates. A simpler readout of conjugation—the OD_{600} in Em + Tm broth—could enable future studies of transposon mutant libraries, small molecule inhibitors, or diverse strain communities. With a focused effort on the

mechanism of pCLJ conjugation, this research could help characterize the dissemination of *bont* genes in nature and reveal new targets for intervention.

Works Cited

1. Pilla G, Tang CM. Going around in circles: virulence plasmids in enteric pathogens. Nat Rev Microbiol. 2018;16(8):484-95. Epub 2018/06/02. doi: 10.1038/s41579-018-0031-2. PubMed PMID: 29855597.
2. Grohmann E, Muth G, Espinosa M. Conjugative plasmid transfer in gram-positive bacteria. Microbiol Mol Biol Rev. 2003;67(2):277-301, table of contents. Epub 2003/06/10. doi: 10.1128/mmbr.67.2.277-301.2003. PubMed PMID: 12794193; PMCID: PMC156469.
3. Lanza VF, Tedim AP, Martinez JL, Baquero F, Coque TM. The Plasmidome of Firmicutes: Impact on the Emergence and the Spread of Resistance to Antimicrobials. Microbiol Spectr. 2015;3(2):PLAS-0039-2014. Epub 2015/06/25. doi: 10.1128/microbiolspec.PLAS-0039-2014. PubMed PMID: 26104702.
4. Adams V, Li J, Wisniewski JA, Uzal FA, Moore RJ, McClane BA, Rood JI. Virulence Plasmids of Spore-Forming Bacteria. Microbiol Spectr. 2014;2(6). Epub 2015/06/25. doi: 10.1128/microbiolspec.PLAS-0024-2014. PubMed PMID: 26104459.
5. Lecuyer F, Bourassa JS, Gelinas M, Charron-Lamoureux V, Burrus V, Beauregard PB. Biofilm Formation Drives Transfer of the Conjugative Element ICE*Bs*1 in *Bacillus subtilis*. mSphere. 2018;3(5). Epub 2018/09/28. doi: 10.1128/mSphere.00473-18. PubMed PMID: 30258041; PMCID: PMC6158512.
6. Lacey JA, Keyburn AL, Ford ME, Portela RW, Johanesen PA, Lyras D, Moore RJ. Conjugation-mediated horizontal gene transfer of *Clostridium perfringens* plasmids in the chicken gastrointestinal tract results in the formation of new virulent strains. Appl Environ Microbiol. 2017. Epub 2017/10/17. doi: 10.1128/AEM.01814-17. PubMed PMID: 29030439; PMCID: PMC5717208.
7. Smillie C, Garcillan-Barcia MP, Francia MV, Rocha EP, de la Cruz F. Mobility of plasmids. Microbiol Mol Biol Rev. 2010;74(3):434-52. Epub 2010/09/02. doi: 10.1128/MMBR.00020-10. PubMed PMID: 20805406; PMCID: PMC2937521.
8. Grohmann E, Keller W, Muth G. Mechanisms of Conjugative Transfer and Type IV Secretion-Mediated Effector Transport in Gram-Positive Bacteria. In: Backert S, Grohmann E, editors. Type IV Secretion in Gram-Negative and Gram-Positive Bacteria. Cham: Springer International Publishing; 2017. p. 115-41.
9. Dunny GM, Berntsson RP. Enterococcal Sex Pheromones: Evolutionary Pathways to Complex, Two-Signal Systems. J Bacteriol. 2016;198(11):1556-62. Epub 2016/03/30. doi: 10.1128/JB.00128-16. PubMed PMID: 27021562; PMCID: PMC4959283.
10. Auchtung JM, Aleksanyan N, Bulku A, Berkmen MB. Biology of ICE*Bs*1, an integrative and conjugative element in *Bacillus subtilis*. Plasmid. 2016;86:14-25. Epub 2016/07/07. doi: 10.1016/j.plasmid.2016.07.001. PubMed PMID: 27381852.
11. Stingl K, Koraimann G. Prokaryotic Information Games: How and When to Take up and Secrete DNA. Curr Top Microbiol Immunol. 2017;413:61-92. Epub 2017/01/01. doi: 10.1007/978-3-319-75241-9_3. PubMed PMID: 29536355.
12. Naglich JG, Andrews RE. Tn*916*-Dependent Conjugal Transfer of pC194 and pUB110 from *Bacillus subtilis* into *Bacillus thuringiensis* subsp. *israelensis*. Plasmid. 1988;20(2):113-26. Epub 1988/09/01. doi: Doi 10.1016/0147-619x(88)90014-5. PubMed PMID: WOS:A1988R376100003.
13. Showsh SA, Andrews RE, Jr. Functional comparison of conjugative transposons Tn*916* and Tn*925*. Plasmid. 1996;35(3):164-73. Epub 1996/05/01. PubMed PMID: 8812783.

14. Showsh SA, Andrews RE, Jr. Analysis of the requirement for a pUB110 mob region during Tn*916*-dependent mobilization. Plasmid. 1999;41(3):179-86. Epub 1999/06/15. doi: 10.1006/plas.1999.1398. PubMed PMID: 10366523.
15. Skarin H, Hafstrom T, Westerberg J, Segerman B. *Clostridium botulinum* group III: a group with dual identity shaped by plasmids, phages and mobile elements. BMC Genomics. 2011;12:185. Epub 2011/04/14. doi: 10.1186/1471-2164-12-185. PubMed PMID: 21486474; PMCID: PMC3098183.
16. Woudstra C, Le Marechal C, Souillard R, Anniballi F, Auricchio B, Bano L, Bayon-Auboyer MH, Koene M, Mermoud I, Brito RB, Lobato FCF, Silva ROS, Dorner MB, Fach P. Investigation of *Clostridium botulinum* group III's mobilome content. Anaerobe. 2018;49:71-7. Epub 2017/12/31. doi: 10.1016/j.anaerobe.2017.12.009. PubMed PMID: WOS:000428487900012.
17. Hatheway CL, McCroskey LM, Lombard GL, Dowell VR, Jr. Atypical toxin variant of *Clostridium botulinum* type B associated with infant botulism. J Clin Microbiol. 1981;14(6):607-11. Epub 1981/12/01. PubMed PMID: 7037830; PMCID: PMC274006.
18. de Jong LI, Fernandez RA, Pareja V, Giaroli G, Guidarelli SR, Dykes JK, Luquez C. First report of an infant botulism case due to *Clostridium botulinum* type Af. J Clin Microbiol. 2015;53(2):740-2. Epub 2014/12/17. doi: 10.1128/JCM.02894-14. PubMed PMID: 25502535; PMCID: PMC4298556.
19. Hosomi K, Sakaguchi Y, Kohda T, Gotoh K, Motooka D, Nakamura S, Umeda K, Iida T, Kozaki S, Mukamoto M. Complete nucleotide sequence of a plasmid containing the botulinum neurotoxin gene in *Clostridium botulinum* type B strain 111 isolated from an infant patient in Japan. Mol Genet Genomics. 2014;289(6):1267-74. Epub 2014/08/26. doi: 10.1007/s00438-014-0887-4. PubMed PMID: 25149145.
20. Zhang S, Masuyer G, Zhang J, Shen Y, Lundin D, Henriksson L, Miyashita SI, Martinez-Carranza M, Dong M, Stenmark P. Identification and characterization of a novel botulinum neurotoxin. Nature communications. 2017;8:14130. Epub 2017/08/05. doi: 10.1038/ncomms14130. PubMed PMID: 28770820; PMCID: PMC5543303.
21. Dover N, Barash JR, Hill KK, Xie G, Arnon SS. Molecular characterization of a novel botulinum neurotoxin type H gene. J Infect Dis. 2014;209(2):192-202. Epub 2013/10/10. doi: 10.1093/infdis/jit450. PubMed PMID: 24106295.
22. Marshall KM, Bradshaw M, Johnson EA. Conjugative botulinum neurotoxin-encoding plasmids in *Clostridium botulinum*. PLoS One. 2010;5(6):e11087. Epub 2010/06/17. doi: 10.1371/journal.pone.0011087. PubMed PMID: 20552020; PMCID: PMC2884020.
23. Bradshaw M, Tepp WH, Whitemarsh RC, Pellett S, Johnson EA. Holotoxin Activity of Botulinum Neurotoxin Subtype A4 Originating from a Nontoxigenic *Clostridium botulinum* Expression System. Appl Environ Microbiol. 2014;80(23):7415-22. Epub 2014/09/23. doi: 10.1128/AEM.01795-14. PubMed PMID: 25239905; PMCID: PMC4249187.
24. Whitemarsh RC, Tepp WH, Johnson EA, Pellett S. Persistence of botulinum neurotoxin A subtypes 1-5 in primary rat spinal cord cells. PLoS One. 2014;9(2):e90252. Epub 2014/03/04. doi: 10.1371/journal.pone.0090252. PubMed PMID: 24587301; PMCID: PMC3937374.
25. Lin WJ, Johnson EA. Transposon Tn*916* mutagenesis in *Clostridium botulinum*. Appl Environ Microbiol. 1991;57(10):2946-50. Epub 1991/10/01. PubMed PMID: 1660696; PMCID: PMC183902.
26. Banuelos-Vazquez LA, Torres Tejerizo G, Brom S. Regulation of conjugative transfer of plasmids and integrative conjugative elements. Plasmid. 2017;91:82-9. Epub 2017/04/26. doi: 10.1016/j.plasmid.2017.04.002. PubMed PMID: 28438469.

27. Johnson EA, Lin WJ, Zhou YT, Bradshaw M. Characterization of neurotoxin mutants in *Clostridium botulinum* type A. Clin Infect Dis. 1997;25 Suppl 2:S168-70. Epub 1997/10/06. PubMed PMID: 9310666.
28. Singh PK, Meijer WJ. Diverse regulatory circuits for transfer of conjugative elements. FEMS Microbiol Lett. 2014;358(2):119-28. Epub 2014/07/06. doi: 10.1111/1574-6968.12526. PubMed PMID: 24995588.
29. Cooksley CM, Davis IJ, Winzer K, Chan WC, Peck MW, Minton NP. Regulation of neurotoxin production and sporulation by a Putative *agrBD* signaling system in proteolytic *Clostridium botulinum*. Appl Environ Microbiol. 2010;76(13):4448-60. Epub 2010/05/11. doi: 10.1128/AEM.03038-09. PubMed PMID: 20453132; PMCID: PMC2897414.
30. Connan C, Popoff MR. Two-component systems and toxinogenesis regulation in *Clostridium botulinum*. Res Microbiol. 2015;166(4):332-43. Epub 2015/01/17. doi: 10.1016/j.resmic.2014.12.012. PubMed PMID: 25592073.
31. Hirt H, Greenwood-Quaintance KE, Karau MJ, Till LM, Kashyap PC, Patel R, Dunny GM. *Enterococcus faecalis* Sex Pheromone cCF10 Enhances Conjugative Plasmid Transfer In Vivo. MBio. 2018;9(1). Epub 2018/02/15. doi: 10.1128/mBio.00037-18. PubMed PMID: 29440568; PMCID: PMC5821081.
32. Stalder T, Top E. Plasmid transfer in biofilms: a perspective on limitations and opportunities. NPJ Biofilms Microbiomes. 2016;2. Epub 2016/01/01. doi: 10.1038/npjbiofilms.2016.22. PubMed PMID: 28480050; PMCID: PMC5416938.