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Historical and current perspectives on *Clostridium botulinum* diversity

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Abstract

For nearly one hundred years, researchers have attempted to categorize botulinum neurotoxin-producing clostridia and the toxins that they produce according to biochemical characterizations, serological comparisons, and genetic analyses. Throughout this period the bacteria and their toxins have defied such attempts at categorization. Below is a description of both historic and current *Clostridium botulinum* strain and neurotoxin information that illustrates how each new finding has significantly added to the knowledge of the botulinum neurotoxin-containing clostridia and their diversity.

Keywords

Clostridium botulinum ; Botulinum neurotoxins; Genomics; Serotype

1. Introduction

The original hypothesis was that botulism was caused by a toxin produced by a single anaerobic spore-forming organism. This bacterium was originally named *Bacillus botulinus* and was later changed to *Clostridium botulinum* when the aerobic *Bacillus* genus was separated from the anaerobic *Clostridium* genus [142]. Almost from the beginning, physiological, biochemical and serological studies indicated an underlying diversity within the botulinum neurotoxin-producing (BoNT-producing) clostridia and their toxins. The original two isolates available for study, the European Van Ermengem and Landmann strains [133,85], differed greatly in growth requirements and biochemical reactions [86]. From these early studies we have been able to hypothesize that the Van Ermengem strain was nonproteolytic, most likely a BoNT/B-producing strain, while the Landmann strain was a proteolytic BoNT/A-producing strain.

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Conflict of interest

The authors declare no conflicts of interest.

Botulism related to contaminated sausages and other meats had been a recognized health hazard in Europe for generations, but it appears its recognition in the United States may have been linked to the widespread acceptance of home and commercial canning methods for food preservation in the early 1900s. An additional difference between European and US strains was recognized among the botulinum neurotoxins due to toxin potency, or toxicity. This difference was especially notable during the “olive scare” of 1919–1920, where four separate botulism outbreaks occurred due to commercially canned ripe olives [4,5,7,114,6]. The mortality rate with these cases was greater than 60%, compared to typical mortalities of approximately 35% or less in Europe. Differences in implicated foodstuffs, such as meats (in Europe) versus vegetables and fruits (in the US), were also noted [27].

2. Botulinum neurotoxin-producing clostridial strains

In 1910–1919, serological methods were introduced for categorizing the toxins or bacteria. These methods were based on the abilities of antisera produced using one toxin or bacterial isolate to neutralize toxins or to agglutinate bacteria of different isolates. The earliest toxin neutralization methods resulted in differentiation of two toxin types, A and B [86,16], while the agglutination methods separated bacterial strains into seven agglutination groups [111]. Agglutination Groups I–III were composed mainly of BoNT/A-producing bacterial strains and Groups IV–VII were associated with BoNT/B-producing strains. Groups I, II, IV, V, and VI contained multiple members, but Groups III and VII contained single members whose antisera was “ultra-specific” for that strain. Several type A Group I strains cross-agglutinated type B Group V strains, and vice versa, indicating that the same bacterial strains could produce toxins of different types, or possibly multiple toxins, and illustrating from the outset that *C. botulinum* strains were diverse and difficult to categorize.

Despite these diversity issues, the *C. botulinum* species continued to be defined on the basis of a single characteristic, the production of botulinum neurotoxin. Adding confusion to the nomenclature was the presence of *C. botulinum* strains that no longer produce neurotoxin, which is a condition that is particularly found in strains that have been repeatedly sub-cultured in the laboratory [55].

In 1947 a simple, rapid method for discriminating BoNT-producing *C. botulinum* strains in mixed cultures was introduced based on differential reactions of colonies on egg yolk agar [92]. Two reactions can be discerned – the lipase reaction which results in an oily sheen on the colonies, and the lecithinase reaction, which causes an opaque white ring in the media below the colonies. *C. botulinum* organisms could be distinguished by their positive lipase and negative lecithinase characteristics. However, as different BoNT-producing clostridia with variable lipase/lecithinase reactions were identified, it became clear that these reactions cannot be depended on alone for the identification of *C. botulinum* strains (Table 1).

Using a combination of biochemical characteristics, it was possible to separate *C. botulinum* strains into four metabolic/biochemical Groups [118,58]. Molecular analyses of *C. botulinum* strains have established that these four Groups represent distinct species, and that these species include nontoxic as well as neurotoxic members [24,67,126]. Additional BoNT-producing clostridial strains (*Clostridium baratii* and *Clostridium butyricum*) have

been identified and characterized [56,93]. Notably, BoNT-producing clostridial strains of *C. baratii* and *C. butyricum* have never been classified as *C. botulinum*, most likely due to fact that these species were already well established. There is also a report of two non-neurotoxigenic *Clostridium subterminale* strains with *bont/B* genes containing a premature stop codon that prevents the expression of BoNT/B, known as silent B genes [43]. It is now evident that multiple clostridial species may produce BoNTs, and the characterization of BoNT-producing clostridia as a single species is erroneous. To a large extent, researchers indirectly acknowledge the presence of multiple species of BoNT-producing clostridia by using the metabolic Group designations in most contemporary publications. Nonetheless, no alternative nomenclature for this group of organisms has been accepted.

3. Botulinum neurotoxins

As noted above, one of the earliest ways to categorize BoNT-producing strains was by serological methods, to include neutralization of toxins using homologous antisera. Initial serological neutralizations were able to clearly distinguish organisms that produce type A from type B toxins [86,16], and this became a hallmark method for toxin differentiation. In 1922, some toxic *C. botulinum* strains were isolated whose toxins were not neutralized by either of the standard anti-A or anti-B antitoxins. Antisera from one of these strains were used to neutralize other similar isolates, which were designated as type C toxin. However, with type C, there were immediate problems in identification. The original type C strains were isolated by Dr. Ida Bengtson and Dr. Robert Graham in 1922 [12] from larvae of the green fly and contents of chicken crops in affected birds. That same year, Dr. Seddon isolated a very similar organism from a fatal case of “midland cattle disease” in Australia [113], which he designated *Bacillus parabotulinus*. Isolates from these cases were sent to the laboratory of Dr. K. F. Meyer, in San Francisco, where the question was asked “are the type C and Seddon strains serologically and toxicologically alike?” The surprising answer was – yes and no. Results from metabolic and biochemical studies indicated that the bacterial strains could not be differentiated [135]. However, neutralization studies using standard methods in guinea pigs showed that they were protected against type C toxin with type C antitoxin, but not with type A, B, or Seddon antitoxin; however, the “Seddon” toxin could be neutralized by both the type C and Seddon antitoxins [100]. Thus, a close relationship was recognized among these toxins, but they could not be serologically classified as the same. The American isolates were designated Cα and the Australian isolates were named Cβ [54]. It was not until many years later and the advent of genetic sequencing that it was determined the strains associated with bird cases were chimeric toxins composed of two thirds BoNT/C and one third BoNT/D (BoNT C/D), which explained the failure to completely neutralize these toxins with heterologous antisera.

Additional cultures of BoNT-producing *Clostridium parabotulinum* strains were isolated in 1920 and 1927, in association with a fatal cattle disease in South Africa. While the organisms resembled type C-producing *C. botulinum* morphologically, their toxins were not neutralized by type C or Seddon antitoxin. Serological testing indicated that the South African strains produced a toxin that was serologically distinct in that the toxin was not neutralized by type A, B, or C antitoxins and, conversely, type A, B, or C antitoxins could not be neutralized by antitoxin produced from the South African strains [94],

therefore it was designated type D. Meyer & Gunnison noted as early as 1929 that, “the immuno-biochemical behavior of the botulinus antigens awaits solution. Then again the relationship of the proteolytic to the nonproteolytic strains, the inter-relation of the four (strain) types, etc., deserve further investigation. The observation that only homologous antitoxin neutralizes the type A and type D toxins, but that occasionally type B and C antiserums exert heterologous action may possess some significance.”

Three additional toxin types have been identified on the basis of serological testing. While botulism associated with fish has been known as early as 1850 [27], it was not definitively identified until nearly a century later. Type E strains were isolated in the 1930s by Russian researchers Kurochkin and Emelyanuck [84]. Shortly after this, Dr. Elizabeth Hazen, from the New York Department of Health, characterized a toxic *C. botulinum* strain that produced a toxin that was not neutralized by type A, B, or C antitoxin [62], Several Russian isolates were sent to Dr. Janet Gunnison at the Meyer laboratory, who confirmed that they were a fifth, serologically distinct toxin serotype [53].

A *C. botulinum* strain producing type F toxin was first identified in connection with a foodborne botulism outbreak involving liver paste on the island of Langeland, in Denmark, in 1958 [95]. Preliminary neutralization experiments using antisera supplied by the Microbiological Research Establishment, Porton, England indicated that this toxin was serologically distinct from type A, B, C, and D, but showed that some neutralization could be obtained using type E antisera in high concentrations.

A type G-producing *C. botulinum* isolate was identified by Gimenez & Ciccarelli from a soil sample in Argentina in 1969 and shown to be serologically distinct using antisera produced at the Center for Disease Control in Atlanta, Georgia [23]. Type G strains are very rare and have only been found in two surprisingly distant locations – Argentina and Switzerland [47,122,123].

While these serological classifications were somewhat exclusive, issues similar to those with type C toxins began to appear. In 1967, Gimenez and Ciccarelli reported on a new type of *C. botulinum* [46]. Studies of this strain, known as strain 84, indicated that there was both type A and type F toxic activity present. However, they could not distinguish whether this toxic activity was due to “a single molecular species or if it is a mixture of types A and F toxins” [48]. It was later determined that this strain produces three distinct toxins, BoNT/A2 and two type F toxin variants (BoNT/F4 and/F5) [77]. Further examples of strains that appeared to produce mixtures containing multiple toxin types included a *C. botulinum* BoNT/Ba-producing strain [60], a BoNT/AB-producing strain [102], and a BoNT/Bf-producing strain [59]. Currently, several examples of strains that produce two toxins (bivalent toxin-producing strains) have been described. These strains produce type A, B or F toxins in combination, often at different concentrations. In these cases the major toxin component is designated by a capital letter and the minor component by a small letter. At about this time, initial reports of isolations of *C. baratii* strains that produced type F toxin [56] and *C. butyricum* strains that produced type E toxins [10] were published.

In 1993 an excellent review entitled “Serological subtypes of botulinal neurotoxins,” written by Gimenez & Gimenez [50], noted that while delineation of BoNTs into seven antigenic types (A-G) is based upon neutralization with specific antiserum, establishing their classification was a matter for discussion. They felt that the lack of a system of definitions for the organisms and a reliable method for serological identification and typing generated major sources of disagreement. They introduced the ideas of “toxin type”, “toxin subtype”, and “intratypic serological variant” to describe monovalent toxin producers, bivalent toxin producers, and toxins that were not neutralized using standard definitions of toxin and antitoxin. Intratypic serological variants showed quantitative differences in their neutralization with a specific antitoxin and these differences could be expressed in terms of serological homology. Using quantitative neutralization assays, “toxin subtypes” (i.e., bivalent toxin producers) could be differentiated from “intratypic serological variants” (i.e., what many now call toxin subtypes) based upon their efficiency of neutralization. When multiple toxins are present, neutralization of the major toxin with specific antitoxin ceases when the minor toxin emerges, so that studies where the toxin concentrations increase will show an abrupt lack of protection. In contrast, with serological variants, the efficiency of neutralization of the toxin will show the same trend throughout the quantitative assessments. An example illustrating intratypic serological variants was shown by the testing of three type F toxins, where strains 160 and 90SL showed sufficient neutralizing ability to be called type F, but cross-neutralization tests using strain-specific sera versus that produced from the reference Langeland type strain revealed a significant amount of intratypic diversity [49]. It is possible that their strains 160 and 90SL might have been BoNT/F4- and/F5-producing strains, which are known to vary genetically from the classic BoNT/F1 Langeland toxin [105].

4. Insights gained from genomic and neurotoxin sequencing efforts

A new era began with the advent of DNA sequencing techniques. Underlying sequence and protein differences responsible for serological diversity could now be explained, providing a greater understanding of this diversity, but also presenting researchers with additional problems in classification. It was found that, despite their conservation of activity with regard to botulism, different BoNT serotypes share surprisingly little genetic identity. Protein (amino acid) differences among the toxin serotypes A-G range from 37.2 to 69.6%, and DNA (nucleotide) differences range from 24.5 to 44.7% [65].

It was known that BoNTs exist in nature as part of a complex of ~2–6 structural proteins whose genes are found in a cluster. DNA sequencing of this area identified two distinctive gene clusters [44]; one cluster contains three hemagglutinin genes, while the alternative gene cluster contains three genes that code for proteins of unknown functions. Toxins have begun to be classified, in addition to serotype, by their accompanying toxin genes, as in *ha*+ (*orf*−) or *orf+* (*ha*−) clusters [73,67]. Sequencing has also revealed underlying insertions, deletions, and recombination events within these gene clusters that may have facilitated toxin gene transfers from strain to strain and contributed to neurotoxin diversity.

Sequencing of neurotoxin genes has also provided genetic confirmation of the existence of multiple *bont* genes within certain *C. botulinum* strains and revealed strains where two

toxin genes were present but only one toxin was produced, due to a mutation introducing a premature stop codon in one of the genes, known as a silent B gene [120,69]. It has also provided evidence of the chimeric nature of the BoNT C/D and D/C toxins and the close genetic relationship of types C and D overall, explaining the serological cross-reactivity issues with these toxin variants [96,138].

A similar issue has arisen recently with the finding of a new bivalent toxin-producing strain, IBCA10–7060. This strain predominantly produces a B2 subtype toxin, with a minor toxin that could be described as a new serotype (H) or as a chimeric A/F toxin [30]. As with the Bengtson and Seddon BoNT/C variants, no standard serotype specific antitoxin could neutralize this new toxin, nor could combinations including anti-BoNT/A, /B, and /F antitoxins. [11]. Antisera produced from this strain can cross-neutralize BoNT/B1 toxins at a 1:80 dilution but it neutralizes its homologous toxins at a 1:500 dilution. These results show similarities to antitoxin testing of BoNT/C and BoNT/F variants [100,49] and point to differences between qualitative versus quantitative results. Gimenez & Ciccarelli noted that the characterizations of strains 160 and 90SL as BoNT/F variants was only possible due to access to antitoxins of sufficient volume and strength to complete quantitative comparisons. While the serological evaluations indicate that this new toxin variant may be a novel serotype, genetic comparisons might characterize the toxin as an A/F mosaic or chimera, similar to the already described C/D and D/C toxins. As sequence information is unavailable for either toxin of this strain, comparisons were made on the basis of somewhat limited published information [30]. Table 2 compares the nucleotide identities of chimeric C and D toxins and the novel toxin with their counterpart serotypes for each toxin domain (light chain domain = LC, N-terminal heavy chain domain = H_N, C-terminal heavy chain domain = H_C). For each domain, nucleotide identities are ~86–99% for similar *bont* sequences and ~56–81% for dissimilar sequences. The variation observed with the novel toxin type versus *bont/A1* and */F5* is similar to the variation seen between *bont D/C* and *bont/C* and */D*. The greatest sequence variation of the novel toxin is observed within the H_N, while this is the most conserved domain among the *bont/C* and */D* genes.

The difficulties encountered with proper designation of this newly isolated toxin have revived older controversies surrounding toxin nomenclature and added additional complications as to whether the BoNTs should continue to be described in terms of serology or whether genetic identities should take precedence.

Through neurotoxin sequencing, there was now a means of definitively differentiating toxins below the serotype level. It was known that certain toxin serotypes, such as types B and F, may be present as “proteolytic” and “nonproteolytic” forms. With the toxins produced by proteolytic strains, cleavage of the progenitor polypeptide into two segments, known as the light and heavy chains, results in increased, or fully active, toxin. On the other hand, toxins produced by nonproteolytic strains, were present, at least in laboratory cultures, as single progenitor toxins showing greatly decreased toxicity, requiring cleavage by additional enzymes for full activation of the toxin. These differences may well be the result of the organism that produces the toxins, and not of the toxins themselves; however, these differences in toxicity were noted early and provided a basis for differentiating these strains. Neurotoxin sequencing provided a method for quantitating the underlying differences in

these genes, and later genomic sequencing highlighted the fact that the *C. botulinum* strains were related not by the toxin types they produced, but rather by their metabolic characteristics, including their absence of the bacterial enzyme capable of cleaving their neurotoxins.

Intratypic serological toxin variants have been previously described, most notably the A2 "Japanese infant botulism toxin variant" [83] and a B2 variant associated with an infant botulism case in Japan [82]. Neurotoxin gene sequencing has enabled a more specific evaluation of the difference between A1 and A2, and B1 and B2 toxins. The A1 and A2 toxins differed by 10.1% in amino acid residues, while the more closely related B1 and B2 toxins differed by 4.4% [65]. These genetically defined differences were linked to serological and functional differences [128,83,82], and have impacted the effectiveness of some medical countermeasures against these toxins [121,137]. Systematic studies have been undertaken to help better determine the diversity within the BoNTs and the impact of this diversity on our ability to treat or protect the public from botulism. These intratypic genetic variants were called "toxin subtypes" which has caused confusion with the Gimenez description of subtype, and they have also been labeled "toxinotype", "subserotype", "toxin variant", or "genetic variant". Early definitions of toxin subtypes as differing by at least 1.9% in amino acid sequence [120] were quickly scrapped in favor of definitions based on nucleotide sequence differences. Currently, no definitive guidelines exist for defining subtypes.

Definitions based on amino acid versus nucleotide sequences reflect the aims of the research. Researchers who study the functional and structural aspects of the toxins, to include toxin activity and treatments or countermeasures, favor definitions based on derived protein sequences. Those who analyze DNA sequences are interested in the underlying mechanisms that enable these sequence differences to occur and evolve. Both types of sequences have been exploited by clinical scientists to produce a variety of detection and diagnostic methods.

As genetic sequencing techniques became more efficient and economical, genetic characterization of the toxin based upon the DNA sequence provided a powerful tool for classification. To date, nearly 40 toxin subtypes/variants have been proposed, mainly based on differences in genetic sequence [119]. Many subtypes vary by less than 10% at the amino acid level, but a few, particularly BoNT/A and/F subtypes, vary by as much as 15.6–36.2%, making them nearly as different as some serotypes. Due to the ease of obtaining sequence information relative to the more challenging serological and protection studies that require the toxin, the vast majority of these subtypes have not been evaluated with regard to quantitative serological differences or potential issues with treatments or countermeasures.

5. Other toxins produced in neurotoxigenic clostridial strains

The presence of multiple toxins having different modes of action is a hallmark in *Clostridium perfringens* and *Clostridium difficile* strains, but it has been thought to be a rare occurrence in BoNT-producing clostridia. In fact, several toxins devoid of neurotoxic activity have been identified in *C. botulinum* strains, particularly among the Group III

clostridia (Table 3). Non-neurotoxigenic toxins have been known to exist in Group III *C. botulinum* strains since the 1970s [74,32]. Multiple hemolytic toxins have also been identified, as well as toxins possessing unique activities. These “hidden toxins” may be directly toxic [57,127] or may contribute to toxicity through synergistic mechanisms [40].

6. Impact of modern characterization techniques on the study of *C. botulinum* diversity

The advent of polymerase chain reaction (PCR) paired with DNA sequencing provided researchers with new molecular tools to study *C. botulinum* and especially the *bont* gene. The first publications of various *bont* gene sequences represented significant discoveries in *C. botulinum* research and required very time-consuming experiments involving radioactive labeling and manual sequence data entry [14,129,140,68,141,61,13,101,139,31,17]. In addition to the *bont* gene sequences, comparisons of 16S *rrn* gene sequences have provided another way to characterize strains in the different *C. botulinum* Groups [24,67]. Results using these methods support Group and species designations of the *C. botulinum* strains that were originally based upon biochemical and microbiological attributes [118,58].

6.1. PFGE and AFLP

Pulsed-field gel electrophoresis (PFGE) has also provided a tool that can be used in multiple laboratories to characterize strains by comparison of DNA fragment sizes from enzyme digested genomic DNAs [63,97,136,130,116,88,8]. Standardization of this technique (for example, using the PulseNet protocol available at http://www.cdc.gov/pulsenet/PDF/c-botulinum-protocol-508c.pdf) has enabled comparisons of multiple strains representing different Groups of *C. botulinum* bacteria from various laboratories throughout the world, and this continues to be a primary technique for typing *C. botulinum* strains. PFGE using undigested DNA coupled with Southern blot hybridization has also been used to identify *bont* locations as being within the chromosome or within toxin-containing plasmids [45,136,143,91]. A disadvantage to widespread use of PFGE is the existence of untypable strains due to the action of extracellular DNAses that degrade the DNA.

Amplified fragment length polymorphism (AFLP) analysis provides an alternative technique to examine and compare *C. botulinum* strains. AFLP is a method involving restriction enzyme-digested genomic DNA, ligation of short linkers, and subsequent PCR amplification of DNA fragments using fluorescent primers [79,80,66,89,26]. Like PFGE, this method can be affected by DNA degradation due to DNAses, but because AFLP relies on amplification of small DNA fragments relative to the fragments separated by PFGE, untypable strains are not typically observed.

6.2. MLST, VNTR, SNP analyses

In 2007 the first finished genomic sequence of a *C. botulinum* strain, BoNT/A-producing ATCC 3502, was released [112]. This was rapidly followed by other complete genomes of serotype A, B, E and F strains [67]. These particular strains were selected for genomic sequencing based upon historical information and use as reference strains in multiple laboratories. Rapid advances in sequencing platforms and chemistry improved efficiency

and lowered costs, which has led to the depositing of additional finished and incomplete (WGS-whole genome shotgun) sequences. Currently, there are 13 finished genomes and 21 WGS genomic sequences available in Gen-Bank. The availability of genomic sequence information has spawned the opportunity to use other molecular techniques such as such as multi-locus sequence typing (MLST) [73,89,88,99], variable number tandem repeat (VNTR) [89,90,42,132], and single nucleotide polymorphism (SNP) analyses [51] to characterize *C. botulinum* strains. Analysis of MLST sequences of housekeeping genes can differentiate strains within a Group or species, as can VNTR and SNP analyses.

6.3. Microarrays and PCR

Genomic sequence data also provided the opportunity to develop microarrays using specially designed oligonucleotides that could quickly differentiate strains based upon the presence or absence of specific genes [106–108,87,18,9,124,134,98]. Microarrays have been designed to identify specific *C. botulinum* Groups or BoNT serotypes and provide information about toxin cluster genes as well as other genes of interest. Similar to other molecular subtyping approaches, DNA microarray data can be used to assess the genetic diversity among multiple strains within a Group. For instance, a Group II subtyping microarray demonstrated that the genomic background of a *C. botulinum* type E strain isolated in Argentina was more similar to Group II type B strains than other type E strains examined [107] and this finding was subsequently confirmed by comparative genome sequence analysis. Nonetheless, it is important to note that strains may contain unique genetic regions that may not be featured among the microarray probes, which are designed using existing genomic sequences.

The availability of a large number of *bont* gene sequences has provided information needed for the development of detection or diagnostic assays using either conventional PCR or real-time PCR techniques [1,103,21,38,110,81,64,3,41,75]. PCR methods, particularly in conjunction with clinical evidence of botulism, provide important presumptive results that can steer laboratory investigations and reduce the demand for extensive mouse bioassay testing of samples.

6.4. Genomic analyses

The completed *C. botulinum* genomes are especially valuable for use in strain comparisons by various bioinformatics analysis methods. The finished genomes can be used as reference sequences against which new WGS sequences can be assembled and compared to identify insertions, deletions, synteny, and recombination events [67,19,115,20,124,65,98,104]. The availability of high-quality finished genomes is critical for accurate mapping efforts using short-reads produced by many current genomic sequencing platforms.

6.5. Mass spectrometry

Sequence information has also enabled the development and use of a protein-based technique for toxin characterizations using mass spectrometry. Toxins in cultures or within complex mixtures are purified and concentrated using antibody-coated beads. The toxin proteins are fragmented using enzymatic digestion, and the fragments are accurately sized using mass spectrometry. A database containing fragment sizing information for known toxin types, derived from toxin DNA sequences, is used to identify the toxin type and/or

subtype. This method has become increasingly valuable for identifying known and novel toxin types, making it a powerful diagnostic and research tool [76].

The application of molecular tools to the study of *C. botulinum* has resulted in the development of DNA- or protein-based techniques that can rapidly detect and characterize BoNTs and *C. botulinum* strains involved in botulism outbreaks. In addition, novel discoveries have resulted from the use of these techniques. PCR, PFGE, AFLP, MLST, VNTR, microarrays and sequencing have not only supported the original observations of *C. botulinum* researchers involving the variation within the bacteria and their toxins, but have also provided insights for the underlying mechanisms for this variation.

7. Mobile genetic elements and horizontal gene transfer

Horizontal transfer of *bont* genes has resulted in the production of the same toxins by organisms representing different *C. botulinum* Groups, and of different toxin types that were produced by the organisms of the same *C. botulinum* Group. For example, BoNT/B is produced by Group I or Group II strains, BoNT/E by Group II and Group VI strains, and BoNT/F by Group I, II and V strains. The mobility of the genes for A, B, and F toxins is also illustrated by the production of each of these toxins by Group I *C. botulinum* strains. In contrast, genes encoding *bont*/C and/D appear to be limited to *C. botulinum* Group III and *bont*/G genes to Group IV *Clostridium argentinense* strains.

In 2007–2008, eight finished *C. botulinum* genomes representing both Group I and Group II strains were made available for examination and analysis. Analysis of the genomes verified that the toxin gene locations could be either within the chromosome and/or within toxin-containing plasmids and also illustrated the non-random location of toxin genes within the strains. For example, with Group I strains, *bont/A* or*/F* genes are found at particular conserved sites within the plasmid or chromosome, while *bont/B* genes are located at alternative conserved sites [67]. Table 4 provides six examples encompassing three toxin serotypes (A, B and E) where the same toxin subtype has both plasmid and chromosomal locations in different bacterial strains. This duality of toxin location and presence in different strains is an indication of the plasticity of the clostridial genomes that provides the opportunity for the toxin to evolve in different strains and environments.

7.1. Plasmids

Plasmids are common within both toxigenic *C. botulinum* strains and closely related nontoxigenic strains, such as *Clostridium sporogenes* and *C. subterminale*. An early study to determine the frequency of plasmids within strains of these three species showed that 40% of the nontoxigenic and 56% of the toxigenic strains tested contained plasmids. The size of the plasmids in these strains ranged from approximately 50–250 kb [125], which is similar to plasmid sizes determined by genomic sequencing of *C. botulinum*. The loss of *bont/B*- and *bont/G*-encoding plasmids upon serial laboratory passage has been documented, indicating that the toxin gene-encoding plasmids may be somewhat unstable [131,36]. This is consistent with the loss of toxicity in serial passage of other *C. botulinum* strains in the laboratory [55], particularly with Group II and III bacteria.

Plasmids likely serve as a vector for toxin gene mobility among some *C. botulinum* strains [117]. For example, a *bont/F5*-containing plasmid was observed in seven isolates that were collected from soil samples from difference provinces in Argentina, as well as from stool samples of individuals with botulism [104,105]. Four of these strains produced only BoNT/F5 and three produced both BoNT/F5 and BoNT/A2. The *bont/F5*-containing plasmid also appears to share a large degree of genetic similarity with the plasmid observed in strain Af84 [29] (which contains *bont/A2* and *bont/F4* genes within its chromosome and the *bont/F5* gene within a plasmid). The appearance of this same toxin gene-containing plasmid within distinct *C. botulinum* strains lends additional support to plasmid-mediated genetic mobility of certain botulinum toxin gene subtypes.

Genomic sequencing has provided insights into the *bont*-containing plasmids. For the first time the sequence of plasmids containing one or more *bont* genes could be analyzed and compared. The *bont*-containing plasmids in Group I strains range from 148 to 266 kb while a much smaller 47 kb plasmid that contains the *bont*/B4 (nonproteolytic B) gene is present within some Group II strains [67]. The plasmids within Group I strains showed regions of synteny, insertions, and deletions; however, no synteny was observed when Group I and Group II plasmids were compared [65].

While interactions between these bacteria and plasmids appear to be Group-specific, experimental evidence suggests otherwise. Group I-associated plasmids containing *bont/A* and *bont/B* genes and Group II-associated plasmids containing *bont/B4* genes have been experimentally introduced into nontoxigenic Group I strains via conjugative transfer [91]. There was a report of a Group II strain (Prevot 59) that contained *bont/B2* genes [66,25]. However, after further assessment it was found that this isolate, which was also labeled as VPI 2,131, is a proteolytic Group I strain. It is believed that this strain was mis-identified during initial strain processing and characterization.

Although the conjugative plasmid experiments showed that transfers of the same genes between different groups were possible, the efficiency of transfer was low (10^{-6} to 10^{-8}). This inefficiency, coupled with possible geographic barriers and other factors, may account for the apparent lack of naturally occurring cross-species conjugative plasmid interactions between the Group I and Group II bacteria. Plasmids that contain *bont/E* [143] and *bont/G* genes [36,144] have yet to be experimentally evaluated in this manner.

7.2. Bacteriophage

Bacteriophages have been recovered from *C. botulinum* Group I strains that produce BoNT/A,/B, and/F; from Group II BoNT/B,/E, and/F-producing strains; and from Group III BoNT/C and/D-producing strains [70,34,115]. These phages appear to be lysogenic [78], and they vary in size and structural characteristics.

Unique to the *C. botulinum* Group III bacteria are the presence of *bont/C* or*/D* genes within bacteriophage DNA. Toxicity is conferred by the presence of the phage, and *C. botulinum* strains have been made nontoxic through elimination, or curing, of phages. Alternatively, nontoxic strains have been made toxic through phage infection or re-infection. Experimentally, phages that carry *bont/C1* genes within their genomes have been isolated

and used to infect *Clostridium novyi* and nontoxic Group III *C. botulinum* strains that have been cured of their phages [71,33,35]. Curing/reinfection cycles may occur naturally [15] and, in addition, lack of toxicity in bacteria due to phage loss after repeated subculture is a common problem among Group III bacteria [55].

Group III bacteriophages contain the largest known temperate phage genomes. Their ~107–203 bp double-stranded DNA genomes are present as a circular plasmid prophage within their host [109]. The phage genomes contain an exceptionally high number of insertion sequences (IS elements), which contributes to sequence diversity within the prophage DNA. It is also possible that the presence of these IS elements in Group III-specific bacteriophages enabled the insertion of the *bont/C* and*/D* genes into the prophage DNA, which was then passed into Group III bacteria. The few *bont*-containing phage DNA genome sequences that are publicly available reveal a wide range in size and significant divergence in phage genome sequence [109]. While the phage DNA genomes do not show a high degree of identity overall, they appear to be composed of mosaic segments, such that certain regions will show high identity with one phage DNA sequence and another region will show identity with a different phage DNA sequence. These mosaic regions within the prophage sequences are most likely the result of recombination events, facilitated by IS elements, among the several phage variants that may inhabit these bacteria. It is thought that the mosaic *bont C/D* and *D/C* genes may have resulted from of this type of recombination event [109].

7.3. IS elements and transposases

IS elements are a type of transposase that have been identified in many bacteria and are present in the regions flanking the toxin gene cluster and sometimes within the toxin gene cluster itself. Most of the IS elements associated with the toxin cluster identified so far are not intact or full length IS elements but are partial degraded genes. One exception is *C. botulinum* strain IBCA10–7060 that contains the novel type H *bont* gene [30]. In this strain the *bont/H* gene cluster is located within a 54 kb region of DNA that is flanked by two direct repeats of intact IS 110 elements. The two IS 110 elements share 99% identity to each other. The direct repeats of the IS 110 elements and their intact nature suggest a relatively recent insertion of the toxin gene cluster in this strain.

Toxin gene mobility can also be attributed to an association with transposases that are not IS elements. One example is the presence of the *bont/E* gene within two different species, *C. botulinum* (Group II) and *C. butyricum* type E (Group VI). Analysis of the genomic sequences revealed that the insertion site that contains the *bont/E* gene in both species appears to be the result of the same type of transposase activity. Interestingly, a *rarA* gene homolog is split and the inserted DNA contains an intact *rarA* gene with the *bont/E* between the split 5′ partial *rarA* gene and 3′ partial *rarA* gene. This method of integration was confirmed in 41 BoNT/E-producing *C. botulinum* and one BoNT/E-producing *C. butyricum* strain [89], as well as a highly divergent BoNT/E-producing *C. botulinum* strain isolated in Argentina [107]. Sequence differences in *rarA* gene homologs among these divergent species and strains indicate the *bont/E* insertions were the result of independent events.

A similar example has been described utilizing the *topB* gene which encodes DNA topoisomerase III, a type of transposase. In five Group II strains, the *bont/F6* gene was

integrated into the chromosome by splitting a *topB* gene homolog and inserting a 34 kb DNA sequence containing a second intact, functional *topB* and the *bont/F6* [20]. Both the *rarA* and *topB* gene insertions illustrate toxin gene mobility by association with transposes.

It appears that the genetic mobility of the toxin genes and their associated gene clusters has been facilitated by their presence within plasmids and phages, and their ability to associate with transposases. One result of this mobility is bacterial strains that contain multiple toxin genes and that may produce two toxins simultaneously, presenting challenges for diagnostic and treatment options. In addition, plasmids and bacteriophages have shown the capability for cross-species transfers of genetic material, including toxin genes, introducing another level of complexity in identification of the causative agents of botulism.

8. Conclusion

The causative agents of botulism have been intensively studied for nearly 120 years. Collection and characterization efforts using state of the art methods for the time have been undertaken by multiple laboratories, most notably the Hooper Foundation laboratories at the University of California, the National Canners Association, Dr. Ivan Hall, and, later, the Anaerobe Laboratory at the Virginia Institute of Technology, the Food and Drug Administration, the Food Research Institute of the University of Wisconsin, and the Centers for Disease Control and Prevention.

The early characterizations of *C. botulinum* strains revealed a heterogeneous species producing a variety of neurotoxins that are conserved in disease process but serologically distinct. Metabolic and biochemical tests were developed that divided the strains into four Groups, while antibody neutralizations have separated the neurotoxins into seven serotypes. Improvements in culture and characterization methods led to identification of additional neurotoxigenic clostridial species and unusual toxin variants.

DNA sequencing introduced an additional level of understanding of bacterial and toxin diversity. Strains that contained multiple neurotoxin genes, chimeric neurotoxin genes, and neurotoxin variants with sequence differences ranging from ~1 to 33% have been identified. These efforts also discovered and/or verified the existence of genes encoding multiple other toxic elements in addition to the neurotoxin genes that reside within these bacteria.

The generation of a variety of strain and neurotoxin toxin sequences has also enabled the development of methods to compare strains within and among Groups. PCR, PFGE, AFLP, MLST, MS, and microarray analyses have been used as aids in the detection and diagnosis of botulism and for comparisons of bacterial and toxin diversity. Such comparisons may provide important information that supports epidemiological investigations associated with botulism outbreaks.

Genomic sequencing has offered detailed information on the bacteria that produce these neurotoxins. Initial sequencing efforts provided finished genomes of reference strains that have proved valuable for use as scaffolds when analyzing short-read draft genomes. Genomic sequencing methods have become more economical and available, and this has enhanced our ability to identify and characterize *C. botulinum* strains.

As more sequence information becomes available, the limitations on our knowledge in this area are shifting from the acquisition of data to the time and expertise needed for analysis of this data. More efficient and accurate analysis methods are being introduced, which are improving our knowledge of the mobility of neurotoxin genes among these bacteria. Historical findings, while limited by the research tools of the day, were able to determine that botulism was caused by a heterogeneous grouping of organisms that produce a diverse array of neurotoxins. Genetic and bioinformatic methods are providing the tools that continue to expand our knowledge and understanding of the underlying mechanisms resulting in this diversity.

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Table 1

Lipase and lecithinase reactions of neurotoxigenic clostridial strains. References are noted in brackets.

Clostridia	Lipase	Lecithinase	Toxins produced	Nontoxic members [67,72]
Group I (*C. botulinum)*	+	−	A, B, F; Ab, Ba, Bf	*C. sporogenes*
Group II (*C. botulinum*)	+	−	B, E, F	*C. taeniosporum* [a]
Group III (*C. botulinum*)	+	+/−	C, C/D, D, D/C	*C. novyi*
Group IV (*C. argentinense*)	−	−	G	*C. argentinense*, *C. subterminale*, *C. hastiforme*
(*C. baratii*)	−	+	F	*C. baratii*
(*C. butyricum)*	−	−	E	*C. butyricum*

[a]The nontoxic members of each group exhibit the same lipase/lecithinase reactions as the neurotoxigenic bacteria, with the exception of *C. taeniosporum*, for which there is no information.

Table 2

Comparisons of cross-serotype chimeric toxins with their parent toxin sero-types. Percentages are nucleotide identities. Comparisons are shown by toxin domain. Light chain (enzymatic) domain = LC, N-terminal heavy chain (translocation) domain = H_N, C-terminal heavy chain (receptor-binding) domain = H_C.

	LC	**H_N**	**H_C**
C/D versus C	**98.0%**	**95.7%**	64.0%
C/D versus D	62.8%	80.5%	**97.8%**
C versus D	63.0%	79.1%	64.0%
D/C versus C	63.2%	79.0%	**86.4%**
D/C versus D	**98.8%**	**96.9%**	63.7%
H versus F5	**~86%**		
H versus F		~77%	
H versus A1-A5			**95.9–97.4%**
A1 versus F5	55.7%	59.7%	66.8%

Percentages in bold type represent the sequences with the highest identities among the domains.

Table 3

Additional toxins that reside within neurotoxigenic *C. botulinum*. These toxins are not neurotoxigenic, but display a variety of alternative activities.

Toxin	Activity	Found in:	Related to:	References
C2 toxin	ADP-ribosylation of actin	*C. botulinum* Group III	ADP-ribosylating toxins *C. perfringens* iota toxin, *C. spiroforme* toxin, *C. difficile* ADP-ribosyltransferase, *B. cereus* vegetative insecticide peptide (VIP)	[2]
C3 exoenzyme	ADP-ribosylation of Rho proteins	*C. botulinum* Group III	ADP-ribosylating toxins *C. limosum* C3lim; *B. cereus* C3cer; *S. aureus* C3 stau1, 2, and3	[39]
Botulinolysin	hemolytic	*C. botulinum* Group II, III	Thiol-activated cytolysins streptolysin O, pneumolysin, listeriolysin O, perfringenolysin O	[57]
Clostridiolysin S	hemolytic	*C. botulinum* Group I, *C. sporogenes*	*Streptolysin S-type toxins in S. aureus*, *B. thuringiensis*, *S. iniae*, *L. monocytogenes*	[52]
Phospholipase C (Cb-PLC)	Zinc metalloprotease	*C. botulinum* Group III	*C. perfringens* alpha toxin, *C. novyi*-PLC, *C. absonum*-PLC, *C. sordelli*- PLC, *C. bifermentans*-PLC	[40]
Cryoprotein	unknown	*C. botulinum* Group IV	May be unique	[22]
Boticin	bacteriocin	Toxic and nontoxic *C. botulinum* Group I, II	May be unique. Unrelated boticins are known to exist within other clostridial species.	[37,28]

Table 4

Toxin subtypes having multiple genetic locations within the chromosome and plasmids. Each toxin subtype may be found either within the chromosome or a mobile genetic element.

Toxin subtype	Strain	Location	Reference
A2	Kyoto-F	chromosome	[67]
	CDC 1436	plasmid	[45]
B1	CDC1632	chromosome	[45]
	okra	plasmid	[67]
B2	CDC 1828	chromosome	[45]
	ISS-333	plasmid	[45]
B3	CDC 816	chromosome	[45]
	ISS-87	plasmid	[45]
B5	CDC 588	chromosome	[45]
	CDC 1436	plasmid	[45]
E1	Beluga	chromosome	[143]
	CB11/1–1	plasmid	[143]