Critical Reviews in Microbiology, 31:11–18, 2005

ISSN: 1040-841X print / 1549-7828 online
DOI: 10.1080/10408410590912952

Taylor & Francis
Taylor & Francis Group

Clostridium botulinum: A Bug with Beauty and Weapon

H. D. Shukla
Center of Marine Biotechnology, University of Maryland Biotechnology Institute, Baltimore, Maryland, USA

S. K. Sharma
US Food and Drug Administration, Center for Food Safety and Applied Nutrition, College Park, Maryland, USA

***Clostridium botulinum*, a Gram-positive, anaerobic spore-forming bacteria, is distinguished by its significant clinical applications as well as its potential to be used as bioterror agent. Growing cells secrete botulinum neurotoxin (BoNT), the most poisonous of all known poisons. While BoNT is the causative agent of deadly neuroparalytic botulism, it also serves as a remarkably effective treatment for involuntary muscle disorders such as blepharospasm, strabismus, hemifacial spasm, certain types of spasticity in children, and other ailments. BoNT is also used in cosmetology for the treatment of glabellar lines, and is well-known as the active component of the anti-aging medications Botox® and Dysport®. In addition, recent reports show that botulinum neurotoxin can be used as a tool for pharmaceutical drug delivery. However, BoNT remains the deadliest of all toxins, and is viewed by biodefense researchers as a possible agent of bioterrorism (BT). Among seven serotypes, *C. botulinum* type A is responsible for the highest mortality rate in botulism, and thus has the greatest potential to act as biological weapon. Genome sequencing of *C. botulinum* type A Hall strain (ATCC 3502) is now complete, and has shown the genome size to be 3.89 Mb with a G+C content of approximately 28.2%. The bacterium harbors a 16.3 kb plasmid with a 26.8% G+C content—slightly lower than that of the chromosome. Most of the virulence factors in *C. botulinum* are chromosomally encoded; bioinformatic analysis of the genome sequence has shown that the plasmid does not harbor toxin genes or genes for related virulence factors. Interestingly, the plasmid does harbor genes essential to replication, including *dnaE*, which encodes the alpha subunit of DNA polymerase III which has close similarity with its counterpart in *C. perfringens* strain 13. The plasmid also contains similar genes to those that encode the ABC-type multidrug transport ATPase, and permease. The presence of ABC-type multidrug transport ATPase, and permease suggests putative involvement of efflux pumps in bacteriocin production, modification, and export in *C. botulinum*. The *C. botulinum* plasmid additionally harbors genes for LambdaBa04 prophage and site-specific recombinase that are similar to those found in the Ames strain of *Bacillus anthracis*; these genes and their products may play a role in genomic rearrangement. Completion of genome sequencing for *C. botulinum* will provide an opportunity to design genomic and proteomic-based systems for detecting different serotypes on *C. botulinum* strains in the environment. The completed sequence may also facilitate identification of potential virulence factors and drug targets, as well as help characterize neurotoxin-complexing proteins, their polycistronic expression, and phylogenetic relationships between different serotypes.**

Keywords *C. botulinum*; Genome; Bioweapon; Botulism; Neurotransmitter

I. INTRODUCTION

Development and use of botulinum toxin as a possible bioweapon (BT) began at least 60 years ago (Smart 1997). A Japanese biological warfare group admitted to feeding cultures of *C. botulinum* to prisoners with lethal effect during that country's occupation of Manchuria in the 1930s (Arnon et al. 2001). Botulinum neurotoxin has been viewed as a potential bioweapon for decades; indeed, when an international arms control team swept Iraq in the mid 1990s, it found that 19,000 liters of serotype A was loaded into warheads (Cohen & Marshall 2001). Botulinum toxin poses a major biological threat because of its extreme potency, lethality, ease of production, and stability in the environment. A single gram of crystalline toxin, evenly dispersed and inhaled, would kill more than 1 million people (Arnon et al. 2001). Botulinum toxin is the deadliest poison available—the LD50 of BoNT in mice has been measured at 1ng/kg (Gill 1982).

Clostridium botulinum is an anaerobic, Gram-positive, spore-forming rod that causes botulism, a severe neurological disease affecting both humans and animals. Botulism typically results from ingestion of food containing botulinum neurotoxin (BoNT) secreted by growing clostridia (Johnson & Bradshaw 2001; Humeau et al. 2000; Davis 2003). The disease may also occur via inhalation of aerosolized toxin, infestation of deep wounds or lacerations, and as a result of gastrointestinal abnormalities (Shapiro et al. 1998; Maksymowych et al. 1999).

Received 10 May 2004; accepted .

Address correspondence to Hem D. Shukla, Center of Marine Biotechnology, University of Maryland Biotechnology Institute, Baltimore, MD 21202. E-mail: shukla@umbi.umd.edu

Abbreviations: BT, bioterrorism; BoNT, botulinum neurotoxin; NAP, neurotoxin associated proteins; HC, heavy chain; LC, light chain.

Although BoNT causes botulism, it also serves as a powerful treatment for a wide array of involuntary muscular disorders. Intramuscular injection of BoNT paralyzes or weakens the injected muscle while leaving other muscles unaffected—a great asset for the management of dystonias, tics, spasticity, and related ailments. BoNT is administered clinically as a complex with neurotoxin-associated proteins (NAPs) that stabilize neurotoxin and prevent onset of botulism in patients.

Seven serotypes (A to G) of *C. botulinum* have been classified by immunological differences in the neurotoxin they produce, as well as by the reaction of each strain to specific antisera. Within the species *C. botulinum*, four distinct phenotypic groups (I–IV) are recognized: group I consists of proteolytic strains that produce one or more BoNT of type A, B, or F; group II strains are nonproteolytic and synthesize a single BoNT of type B, E, or F; group III strains produce BoNT/C or BoNT/D; and group IV contains strains that produce BoNT/G (Lindström et al. 2001). Different serotypes (A–F) of *C. botulinum* cause botulism in humans and animals (Table 1). While disease correlation for serotype G has not been established, this strain may be associated with human botulism (Shapiro et al. 1998; Yongtai et al. 1995). However, Phylogenetic analyses based on the BoNT protein sequence alignment using CLUSTAL_X 1.81, have suggested that seven serotypes of *C. botulinum* form two major clades (Figure 1). The analysis has shown that clade one has two subclusters, showing phylogenetic relationship between neurotoxin A and E. The other sub cluster exhibited close relationship with serotype C and D. In another major clade serotype F, G, and B showed close relations with strong bootstrep support.

Spores are heat-resistant and can survive in foods that are incorrectly or minimally processed under anaerobic conditions. Germinating spores secrete neurotoxin, which blocks release of acetylcholine at the neuromuscular junction and causes acute flaccid muscle paralysis.

This review explores the detail characteristics of botulinum neurotoxins, current status in vaccine developments against different serotypes of *C. botulinum* and therapeutic uses. It first discusses the types of botulism and their symptoms, followed by the organization and regulation of neurotoxin genes (*bont*), nontoxic-nonhemagglutinin (*ntnh*) genes, and hemagglutinin (*ha*) components and their roles. Next, it delves into the structure and mechanism of action of BoNT, the treatment of botulism via antibody-based therapy, and progress in vaccine development. Finally, it discusses design of a clostridial detection system based on genome sequence, specific proteomic signatures, future prospects of post genomic analysis, and the pharmaceutical applications of BoNT.

TABLE 1
Different serotypes (A–G) of *Clostridium botulinum*, their host, substrate specificity, and specific cleavage site in the substrate

Serotype	Host	SNARE protein (substrate)	Cleaving site
Type A	Human	SNAP-25	Gln^{197}-Arg^{198}
Type B	Human	Syneptobrevin	Gln^{76}-Phe^{77}
Type C	Animals	SNAP-25, Syntaxin	SNAP25 Arg^{198}-Ala^{199}, Syntaxin Lys^{253}-Ala^{254}
Type D	Animals	Syneptobrevin	Lys^{59}-Leu^{60}
Type E	Human, Fish	SNAP25	Arg^{180}- Ile^{181}
Type F	Human	Syneptobrevin	Gln^{58}-Lys^{59}
Type G	Human	Synaptobrevin	Ala^{81}-Ala^{82}

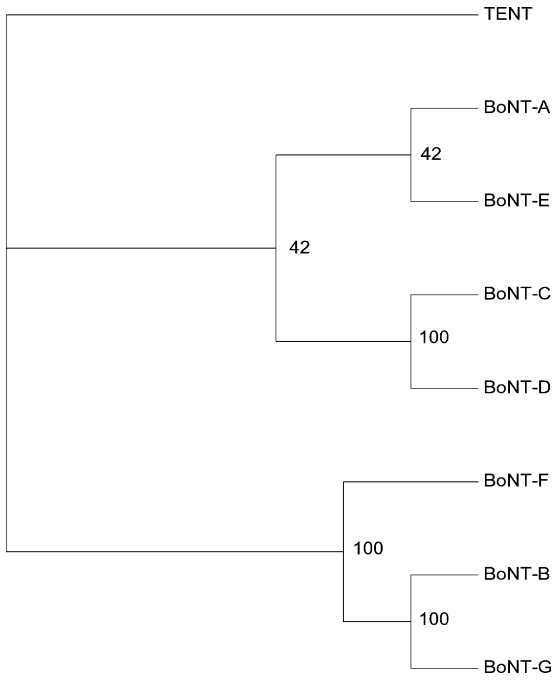

FIG. 1. Neighbor-joining phylogenetic tree of botulinum neurotoxins (BoNT) in different serotypes of *C. botulinum*. Evolutionary relationship among different serotypes of *C. botulinum*. Included in the tree are BoNT homologs from *C. botulinum* serotypes A, B, C, D, E, F, and G respectively, tetanus neurotoxin is included as an outgroup. Numbers at nodes are percentage bootstrapping values.

II. BOTULISM

A. Types of Botulism

1. Wound Botulism

BoNT/A is the major causative agent of wound botulism; however, BoNT/B has also been associated with development of the disease (Shapiro et al. 1998). Because *C. botulinum* undergoes anaerobic spore formation, deep wounds are especially conducive to proliferation of the organism and toxigenesis. Wound botulism typically occurs in cases of deep subcutaneous lacerations, such as those associated with intravenous drug use.

2. Infant Botulism

Approximately half of all cases of infant botulism are attributable to BoNT/A, while the other half are attributable to

BoNT/B. Infant botulism results from ingestion of *C. botulinum* spores that colonize the gastrointestinal tract and secrete neurotoxin, which is then absorbed from the bowel lumen. Infant botulism affects children less than six months old, presumably due to either immature gut physiology or inadequate development of the gut flora, which serve as a defense against the disease in older children (Armada et al. 2003). A link between infant botulism and sudden infant death syndrome (SIDS) has been proposed because of a similarity between the sudden respiratory arrest observed in both diseases (Midura 1996).

3. Food-Borne Botulism

Like infant botulism, food-borne botulism occurs when *C. botulinum* colonizes the gastrointestinal tract and secretes neurotoxin. Although this type of botulism is mainly transmitted through improperly stored food containing BoNT, it has also been linked to gastrointestinal tract abnormalities as well as disruption of the natural gastrointestinal flora by antibiotic treatment (Shapiro et al. 1998; Kobayashi et al. 2003).

4. Inhalational Botulism

Air-borne botulinum toxin can interact with the respiratory system and cause inhalational botulism (Park & Simpson 2003). Studies in monkeys indicate that, if aerosolized, botulinum toxin also can be absorbed through the lungs (Arnon et al. 2001). This mode of transmission has been used in the past as bioweapon (Tucker 2000).

B. Symptoms

Classic symptoms of botulism include blurred vision, drooping eyelids, slurred speech, difficulty swallowing, dry mouth, and muscle weakness. Infants with botulism appear lethargic, feed poorly, are constipated, and have a weak cry and poor muscle tone. Left untreated, these symptoms may progress to cause paralysis of the arms, legs, trunk and respiratory muscles. In food-borne botulism, symptoms generally appear 18 hours to 36 hours following ingestion of contaminated food; however, onset may occur as early as 6 hours later, or as late as 10 days after a meal (Shapiro et al. 1998; Woodruff et al. 1992; Hatheway 1995). People exposed to the toxin require immediate and intensive treatment. Onset time and severity of symptoms depends on the amount and rate of toxin absorbed by the circulatory system. Symptoms subside when new motor axon twigs re-enervate paralyzed muscles.

III. ORGANIZATION, STRUCTURE AND MECHANISM OF ACTION OF BOTULINUM NEUROTOXIN AND ITS THERAPEUTIC USES

A. Organization and Regulation of Neurotoxin Genes

Botulinum neurotoxins associate with non-toxic clostridial proteins to form large, stable complexes that exist in cultures known as progenitor toxins. These progenitor toxins comprise three different forms: 12S (C 300 kDa), 16S (C 500 kDa), and 19S (C 900 kDa), and consist of neurotoxin subunits coupled with one or more non-toxic components known as neurotoxin associated proteins (NAPs). NAPs possess hemagglutinin activity (HA) protect the neurotoxin from harsh environment inside host like low pH and proteases, and include HA-17, HA-22, HA-33, and HA-55 (Sagane et al. 2001; Shukla et al. 1997; Chen et al. 1998). Recent reports suggest that HA-33 in type A neurotoxin complex, enhanced BoNT catalytic activity by 25-fold (Sharma & Singh 2004). As discussed later in this review, NAPs also provide a vehicle for the safe clinical use of BoNT.

FIG. 2. Organization of neurotoxin (*bont*), nontoxic-nonhemagglutinin (*ntnh*), and hemagglutinin (*ha*) genes in the chromosome of *C. botulinum* serotype A; *botR* is a positive regulator of *bont* genes.

Genes encoding BoNT and its associated proteins are localized at different positions in the *C. botulinum* genome for different clostridial serotypes. For serotype A, the *bont* gene and NAP genes are clustered together at the botulinum locus (Figure 2). Comparative analyses of the BoNT locus have been performed extensively, and have revealed interesting phylogenetic relationships both within and among the different toxin serotypes (Popoff & Marvaud 1999). Neurotoxin genes for serotypes A, B, E, and F are nested within the chromosome; for serotypes C and D, these genes are located in phage DNA. BoNT genes for serotype G are positioned in a large plasmid similar to that found in *C. tetani* (Marvaud et al. 1998, 2000). The gene encoding nontoxic-nonhemagglutinin (NTNH) components is localized immediately upstream of the *bont* gene in serotype A; both genes transcribe in the same orientation. In serotypes A, B, C, and G, genes for HA components are present upstream of, and are transcribed in opposition to, the *ntnh* gene and the *bont* gene (Marvaud et al. 2000; Popoff & Marvaud 1999; Dineen et al. 2003). Transcription of neurotoxin is under positive control of the regulator gene *botR* (Figure 1). The product of *botR* is a 21 kDa, conserved protein with 67% identity to the *tetR* gene product in *C. tetani*, and almost 30% identity with UviA, a putative activator of bacteriocin in *C. perfringens* (Marvaud et al. 1998).

B. BoNT: Structure and Mechanism of Action

The seven immunologically distinct botulinum neurotoxins (BoNT/A–BoNT/G) are homologous proteins consisting of a heavy chain and light chain linked by essential disulfide and noncovalent interactions that specifically block release of acetylcholine at the neuromuscular junction (Smart 1997). Botulinum neurotoxin is initially synthesized as a 150 kDa single-chain protein that is post-translationally nicked to form a dichain structure. The nicked neurotoxin consists of a 100 kDa heavy chain and a 50 kDa light chain (Zinc metalloprotease) linked by a disulfide bridge (Figure 3) (Yowler et al. 2002). The crystal structure of BoNT indicates that the heavy chain consists of a binding domain (H_C) and a translocation domain (H_N), while the light chain contains a catalytic domain (L_C) (Swaminathan & Eswaramoorthy 2000). The H_C domain (comprising two subdomains of equal

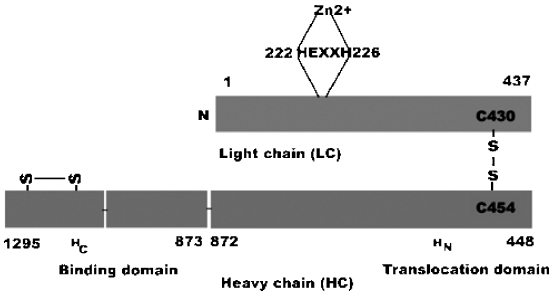

FIG. 3. Structure of botulinum neurotoxin type A, exhibiting heavy and light chain and their different domains.

size) binds to receptors on the cell surface, and the H_N domain mediates translocation of the L_C domain across the cell membrane (Schiavo et al. 2000; Turton et al. 2002).

Recent reports suggest that the acidic pH of the clostridial endosome partially unfolds the light chain and allows its translocation through the H_C channel, which acts as a transmembrane chaperone (Koriazova & Montal 2003). Additional studies have shown that there is an extremely conserved region (called HEXXH) among the light chains of all the BoNTs that is typical of zinc-binding motifs of zinc endoproteinases (Swaminathan & Eswaramoorthy 2000). After internalization of the light chain and translocation into the cytosol, HEXXH catalyses the proteolysis of SNARE protein complexes involved in exocytosis of synaptic vesicles containing acetylcholine (Figure 4A, B). Hence, the release of acetylcholine at the neuromuscular junction is blocked, leading to muscle paralysis and flaccidity. The seven BoNTs exhibit somewhat different protease activities, cleaving three SNARE proteins (synaptobrevin/VAMP, SNAP-25, and syntaxin) at specific amino acid sites (Table 1). Ca 2+ plays important role in the process of neurotoxin inhibition of neurotransmitter release (Meunier et al. 2002).

C. Treatment: Antibody-Based Therapy

Passive immunotherapy has been established as a valuable prophylactic and effective post-exposure approach to botulinum toxicity (Mayers et al. 2001). The specific treatment is based on serotherapy or antibody-based therapy—currently, both equine antitoxin and human botulinum immune globulin are being used to treat adult and infant botulism, respectively (Franz et al. 1993; Nowakowski et al. 2002; Arnon 1993). A licensed trivalent antitoxin that contains neutralizing antibodies against botulinum toxin types A, B, and E and an investigational heptavalent (A–G) antitoxin are also being used in antibody-based therapy (Arnon et al. 2001; Hibbs et al. 1996). Recently, it has been shown that type A neurotoxin can be potentially neutralized by an oligoclonal Ab consisting of only three monoclonal antibodies. This finding could provide a research route to drugs for preventing and treating botulism, as well as diseases caused by other pathogens and biologic threat agents (Nowakowski et al. 2002). However, development of broadly protective monoclonal antibodies is dependent on understanding the diversity between representative clinical and environmental isolates and their botulinum neurotoxins. The priority for development of each monospecific product, from highest to lowest, is serotype A, B, E, C, F, G, and D.

D. Progress in Vaccine Research

The potential use of BoNT as a bioweapon has increased the need for an effective vaccine against botulism (Shoham 2000; Bennett et al. 2003). Botulinum toxoid has been successfully used for vaccination, and an effective tetravalent toxoid (for serotypes A, B, E, and F) has been developed for protecting neurotoxin researchers in Japan (Montgomery et al. 2000; Torii et al. 2002). A pentavalent vaccine, produced using a formalin-inactivated culture supernatant from *C. botulinum*, was designed to immunize military personnel from biological weapons containing serotypes A, B, C, D, and E (Bennett et al. 2003). However, it requires frequent boosters to maintain protective levels, is associated with side effects, and does not provide immunity against serotypes F and G. A bivalent recombinant vaccine has been developed to protect against serotypes C and D, and has shown protective effects in both humans and animals (Woodward et al. 2003). A recent report suggests that a heavy chain (H_C) vaccine would be more protective than a toxin-based vaccine (L_C), since more H_C antibodies are generated to block cellular receptors (Amersdorfer et al. 2002).

DNA-based vaccines have recently generated great interest because of their manufacturing simplicity, purity of product, and ease of storage. Generation of DNA vaccines does not require pathogen culturing, and is therefore safer than manufacture of toxoid vaccines. In addition, DNA vaccines impart immunity via production of recombinant H_C protein within cells of the vaccinee, so that protein purification is not required. A major advantage of DNA vaccines is flexibility and a short timeframe of development (Bennett et al. 2003). However, past attempts at immunization via DNA encoding of the BoNT/A H_C domain have met with limited success because of a low immunity rate compared with toxoid or protein fragment vaccination (Clayton & Middlebrook 2000). However, recent research has demonstrated that addition of a signal sequence to the DNA vaccine against serotype F enhances antibody response to the recombinant FH_C domain (Bennett et al. 2003). This signal modification technique has the potential to make DNA vaccination against botulinum toxin a clinically viable approach.

E. The Post-Genomic Future of *C. botulinum* Research

Genome sequencing of *C. botulinum* genome has been completed at the Wellcome-Trust Sanger Institute, UK [http://www.sanger.ac.uk/Projects/C_botulinum/]. The genome of *C. botulinum* Hall strain A (ATCC 3502) is 3.89 Mb in size, with a G+C content of approximately 28.2%. Virulence factors of *C. botulinum* are primarily encoded within the chromosome, in contrast to other clostridia such as *C. tetani*, which harbors genes for tetanus toxin (*tet*X) on its 74 kb plasmid (pE88). The *C. botulinum* genome has a two-component regulatory system

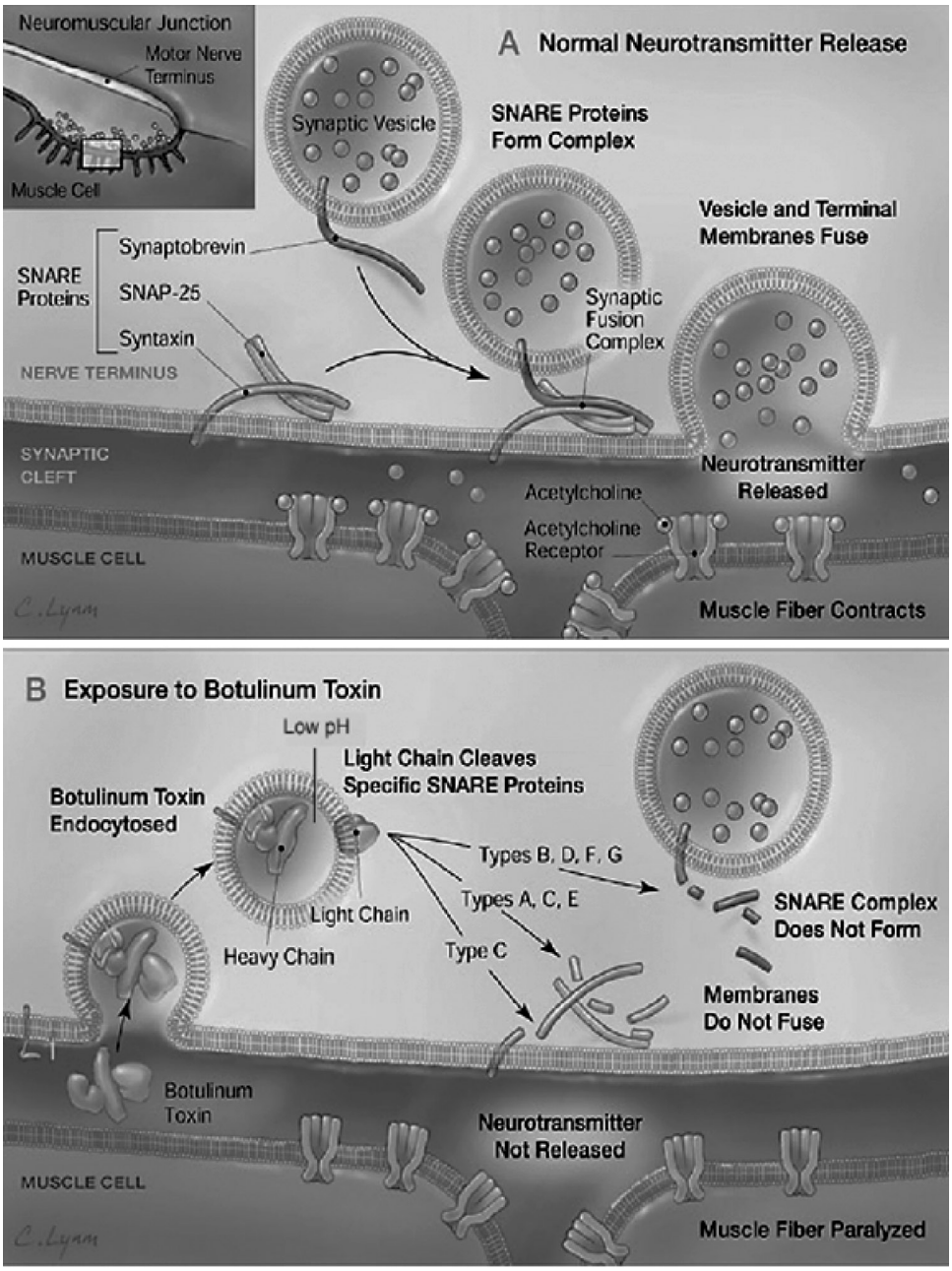

FIG. 4. (A) Release of acetylcholine at the neuromuscular junction is mediated by the assembly of a synaptic fusion complex that allows the membrane of the synaptic vesicle containing acetylcholine to fuse with the neuronal cell membrane and results in muscle contraction. (B) Botulinum toxin binds to the neuronal cell membrane at the nerve terminus and enters the neuron by endocytosis. The light chain of botulinum toxin cleaves specific sites on the SNARE proteins, preventing complete assembly of the synaptic fusion complex and thereby blocking acetylcholine release.

and surface layer proteins (SLP) that have closest homology to those of *C. tetani*. *C. botulinum* type A also harbors one 16.3 kb plasmid that has 26.8% G+C content (slightly lower than that of the chromosome).

Analysis has shown that the *C. botulinum* plasmid does not harbor genes for toxin or related virulence factors. Interestingly, the plasmid does harbor genes essential to replication, including those for the alpha subunit of DNA polymerase III (*dnaE*). Genes within the plasmid bear close similarity to those that encode the ABC-type multidrug transport system, ATPase, and permease components of plasmid 13 in *C. acetobutylicum*. This finding suggests putative involvement of efflux pumps in bacteriocin production, modification, and export. The plasmid also harbors genes for LambdaBa04 prophage and site-specific recombinase may have role in gene transfer (Canchaya et al. 2003). In addition, the *C. botulinum* plasmid contains a protease gene

similar to that of *Fusobacterium nucleatum*, an oral bacterium that breaks down excess protein in the human mouth (Kapatral et al. 2002).

The genome sequence of *C. botulinum* type A will facilitate understanding of the organization, regulation, and evolutionary history of the entire neurotoxin gene cluster. Data generated also may aid vaccine development by shedding light on the mechanism of early-stage toxico-infection, as well as identifying receptors that interact with the BoNT H_C binding domain. Serotype identification via DNA and protein signatures is another potential benefit of post-genomic analysis; combined with proteomic analysis, genomic analysis could pinpoint other virulence factors involved in botulism disease. Since, the complete genome sequence allows analysis of unique intergenic sequences, it may aid in the development of a sensitive system for detecting different serotypes of *C. botulinum* in the environment. Studies in comparative genomics with other pathogens such as *C. perfringens* and *C. tetani* will provide a unique opportunity to investigate the phylogenetic relationship between different species of clostridia.

F. Pharmaceutical Applications of Neurotoxin and Its Complex

Clostridial neurotoxins and their associated proteins are a rich repository for research and therapeutic applications (Brinn 1997; Kennedy 2002; Rosetto et al. 2001). The pharmaceutical significance of botulinum neurotoxin lies in its capacity to inhibit involuntary muscle movement over a stipulated period of time. Currently, botulinum neurotoxin A and B are available for clinical use, and have been shown to be safe and effective for the treatment of various kind of muscle disorders. One study conducted in humans with dystonias has shown that, among three toxins, BoNT/A causes the longest interval of neuromuscular paralysis (four months), followed by BoNT/B and BoNT/E (Foran et al. 2003). While the impact of BoNT/C on neuromuscular paralysis is not well established, its duration of neuroparalytic impact may be similar to BoNT/A (Foran et al. 2003). The Food and Drug Administration (FDA) has approved an injectable preparation of BoNT/A for the treatment of strabismus, blepharospasm, hemifacial spasm, and certain types of spasticity in children (Munchau & Bhatia 2000). This clinical formulation comprises BoNT/A complexed with other, non-toxic clostridial proteins that stabilize the labile neurotoxin after administration, and may also slow its diffusion into affected tissues. As is the case for botulism, the toxin binds to nerve endings and inhibits release of acetylcholine that would otherwise signal the muscle to contract. Thus, injections with BoNT/A complex block extra contraction [of the muscle] but leave enough strength for normal use. Other investigational uses include: spasmodic dysphonia (which results in speech that is difficult to understand), urinary bladder muscle relaxation (such as in cases where muscle contraction is severe enough to require catheterized urination), esophageal sphincter muscle relaxation, and the management of tics (Munchau & Bhatia 2000). BoNT/A complex is also being used as anti-aging agent in the form of BOTOX and Dysport® (Moore 2002), and research is underway to use clostridial toxins or toxin domains for implementation of a pharmaceutical drug delivery system (Goodnough et al. 2002). Recent reports have suggested that botulinum neurotoxin is quite effective in the treatment of spasticity in HIV-infected children affected with progressive encephalopathy (Noguera et al. 2004).

IV. CONCLUSIONS

Although *Clostridium botulinum* and its neurotoxins are a considerable threat to humans and animals, research has shown that a bug of dangerous weaponry can also become a bug of beauty. Botulinum neurotoxins have generated immense interest within scientific and medical communities because of their value as research tools and therapeutic agents. The complete genome sequence of *C. botulinum* will help researchers identify cellular receptors that interact with the BoNT H_C binding domain and allow L_C traslocation across endosomal membrane. The sequence data will also aid in designing effective botulism vaccines, post-exposure therapies, and a sensitive environmental detection system against all clostridial serotypes.

ACKNOWLEDGEMENTS

We would like to thank Dr. J. Parkhill, Wellcome-Trust Sanger Institute, UK for fruitful discussions and many genome projects that keep their data freely available—they provide a tremendous service to the scientific community. I also thank my colleague Kimberly Polumbo and J. Soneja for their technical assistance in producing this review.

REFERENCES

Amersdorfer, P., Wong, C., Smith, T., Chen, S., Deshpande, S., Sheridan, R., and Marks, J.D. 2002. Genetic and immunological comparison of anti-botulinum type A antibodies from immune and nonimmune human phage libraries. *Vaccine* 20, 1640–1648.

Armada, M., Love, S., Barrett, E., Monroe, J., Peery, D., and Sobel, J. 2003. Foodborne botulismin a six-month-old infant caused by home-canned baby food.*Annual Emergency Medicine* 42, 226–229.

Arnon, S.S. 1993. Clinical trial of human botulism immune globulin. In *Botulinum and Tetanus Neurotoxins: Neurotransmission and Bio-Medical Aspects*, ed B.R. DasGupta, 477–482. New York: Plenum Press.

Arnon, S.S., Schechter, R., Inglesby, T.V., Henderson, D.A., Bartlett, J.G., Ascher, M.S., Eitzen E, Fine, A.D., Hauer, J., Layton, M. et al. 2001.Botulinum Toxin as Biological weapon. *Journal of American Medical Association* 285, 1059–1070.

Bennett, A.M., Perkins, S.D., and Holley, J.L. 2003. NA vaccination protects against botulinum neurotoxin type F. *Vaccine* 21, 3110–3117.

Brinn, M.F. 1997. Botulinum neurotoxin: Chemistry, pharmacology, toxicity, and immunology. *Muscle and Nerve* 20, S146–S168.

Canchaya, C., Fournous, G., Chibani-Chennoufi, S., Dillmann, M.L., and Brussow, H. 2003. Phage as agents of lateral gene transfer. *Current Opinion in Microbiology* 6, 1–8.

Chen, F., Kuziemko, G.M., and Stevens, R.C. 1998. Biophysical characterization of the stability of the 150-kilodalton botulinum toxin,the nontoxic component, and the 900-kilodalton botulinum toxin complex species. *Infection Immunity* 66, 2420–2425.

Clayton, J., and Middlebrook, J.L. 2000. Vaccination of mice with DNA encoding a large fragment of botulinum neurotoxin serotype A. *Vaccine* 18, 1855–1862.

Cohen, J., and Marshall, E. 2001. Vaccines for Biodefense: A system in Distress. *Science*. 294, 498–501.

Davis, L.E. 2003. Botulism. *Current Treatment Options in Neurology* 5, 23–31.

Dineen, S.S., Bradshaw, M., and Johnson, E.A. 2003. Neurotoxin gene clusters in *clostridium botulinum* type A strains: Sequence comparison and evolutionary implications. *Curr. Microbiol.* 46, 345–352.

Foran, P.G., Mohammed, N., Lisk, G.O., Nagwaney, S., Lawrence, G.W., Johnson, E., Smith, L., Aoki, K.R., and Oliver Dolly, J. 2003. Evaluation of the therapeutic usefulness of botulinum neurotoxin B, C1, E, an F Compared with the Long Lasting Type A. *Journal of Biological Chemistry* 278, 1363–1371.

Franz, D.R., Pitt, L.M., Clayton, M.A., Hanes, M.A., and Rose, K.J. 1993. Efficacy of prophylactic and therapeutic administration of antitoxin for inhalation botulism. In *Botulinum and Tetanus Neurotoxins: Neurotransmission and Biomedical Aspect*s, ed. B.R. DasGupta, 473–476. New York: Plenum Press.

Gill, M.D. 1982. Bacterial toxins: A table of lethal amounts. *Microbiological Review*, 46, 86–94.

Goodnough, M.C., Oyler, G., Fishman, P.S., Johnson E.A., Neale, E.A., Keller, J.E., Tepp, W.H., Clark, M., Hartz, S., and Adler, M. 2002. Development of a delivery vehicle for intracellular transport of botulinum neurotoxin antagonists. *FEBS Lett*. 513, 163–168.

Hatheway, C.L. 1995. Botulism: The present status of the disease. *Curr. Top. Microbiology and Immunology* 195, 55–75.

Hibbs, R.G., Weber, J.T., Corwin, A., Allos, B.M., Abdel Rehim, M.S., Sharkawy, S.E., Sarn, J.E., and McKee, K.T. Jr. 1996. Experience with the use of an investigational F (ab')2 heptavalent botulism immune globulin of equine origin during an outbreak of type E botulism in Egypt. *Clin. Infect. Dis.* 23, 337–340.

Humeau, Y., Doussau, F., Grant, N.J., and Poulain, B. 2000. How botulinum and tetanus neurotoxins block neurotransmitter release.*Biochimie* 82, 427–446.

Johnson, E.A., and Bradshaw, M. 2001. Clostridium botulinum and its neurotoxins: A metabolic and cellular perspective. *Toxicon* 39, 1703–1722.

Kapatral, V., Anderson, I., Ivanova, N., Reznik, G., Los, T., Lykidis, A., Bhattacharyya, A., Bartman, A., Gardner, W., Grechkin, G. et al. 2002. Genome sequence and analysis of the oral bacterium *Fusobacterium nucleatum* strain ATCC 25586. *J. Bacteriol*. 184, 2005–2018.

Kennedy, D. 2002. Beauty, biological weapons, and Botox. *Science* 295, 1601.

Kobayashi, H., Fujisawa, K., Saito, Y., Kamijo, M., Oshima, S., Kubo, M., Eto, Y., Monma, C., and Kitamura, M.A. 2003. Botulism case of a 12-year-old girl caused by intestinal colonization of *Clostridium botulinum* type AB. *Japanese Journal of Infectious Diseases* 56, 73–74.

Koriazova, L.K., and Montal, M. 2003. Translocation of botulinum neurotoxin light chain protease through the heavy chain channel. *Nat. Struct. Biol.* 10, 13–18.

Lindström, M., Keto, R., Markkula, A., Nevas, M., Hielm, S., and Korkeala, H. 2001. Multiplex PCR Assay for Detection and Identificationof *Clostridium botulinum* Types A, B, E, and F in Food and Fecal Material. *Applied Environmental Microbiology* 67, 5694–5699.

Maksymowych, A.B., Reinhard, M., Malizio, C.J., Goodnough, M.C., Johnson, E.A., and Simpson, L.L. 1999. Pure botulinum neurotoxin is absorbed from the stomach and small intestine and produces peripheral neuromuscular blockade.*Infection Immunity* 67, 4708–4712.

Marvaud, J.C., Gibert, M., Inoue, K., Fujinaga, V., Oguma, K., and Popoff, M.R. 1998. *bot*R is a positive regulator of botulinum neurotoxin and associated non toxic protein genes in *Clostridium botulinum* A. *Mol. Microbiol.* 29, 1009–1018.

Marvaud, J.C., Raffestin, S., Gibert, M., and Popoff, M.R. 2000. Regulation of the toxinogenesis in *Clostridium botulinum* and *Clostridium tetani*. *Biology of the Cell* 92, 455–457.

Mayers, C.N., Holley, J.L., and Brooks, T. 2001. Antitoxin therapy for botulinum intoxication. *Rev. Med. Micro.* 12, 29–37.

Meunier, F.A., Schiavo, G., and Molgo, J. 2002. Botulinum neurotoxins: From paralysis to recovery of functional neuromuscular transmission. *Journal of Physiology–Paris* 96, 105–113.

Midura, T.F. 1996. Update: Infant Botulism. *ClinicalMicrobiology Review* 9, 119–125.

Montgomery, V.A., Makuch, R.S., Brown, J.E., and Hack, D.C. 2000. The immunogenicity in humans of a botulinum type F vaccine. *Vaccine* 18, 728–735.

Moore, A. 2002. The biochemistry of beauty. *EMBO Reports* 3, 714–716.

Munchau, A., and Bhatia, K.P. 2000. Uses of botulinum toxin injection in medicine today. *BMJ* 320, 161–165.

Noguera, A., Perez-Duenas, B., Fortuny, C., Lopez-Casas, J., and Poo-Arguelles, P. 2004. Botulinum toxin in the treatment of spasticity in HIV-infected children affected with progressive encephalopathy. *AIDS* 18, 352–353.

Nowakowski, A., Wang, C., Powers, D.B., Amersdorfer, P., Smith, T.J., Montgomery, V.A., Sheridan, R., Blake, R., Smith, L.A., and Marks, J.D. 2002. Potent neutralization of botulinum neurotoxin by recombinant oligoclonal antibody. *Proc. Natl. Acad. Sci. USA* 99, 11346–11350.

Park, J.B., and Simpson, L.L. 2003. Inhalational poisoning by botulinum toxin and inhalation vaccination with its heavy-chain component. *Infection Immunity* 71, 1147–1154.

Popoff, M.R., and Marvaud, J.-C. 1999. *Structural and Genomic Features of Clostridial Neurotoxins*. In *The Comprehensive Sourcebook of Bacterial ProteinToxins*, eds. J.E. Alouf and J.H. Freer, 174–201. London: Academic Press.

Popoff, M.R., and Marvaud, J.-C. 1999. Structural and genomic features ofclostridial neurotoxins. In *The Comprehensive Sourcebook of Bacterial ProteinToxins*, 2nd edn., eds. J.E. Alouf and J.H. Freer, 174–201. London: Academic Press.

Rosetto, O., Seveso, M., Caccin, P., Schiavo, G., and Montecucco, C. 2001. Tetanus and botulinum neurotoxins: Turning bad guys into good by research. *Toxicon* 39, 27–41.

Sagane, Y., Kouguchi, H., Watanabe, T, Sunagawa, H., Inoue, K., Fujinaga, Y., Oguma, K., and Ohyama, T. 2001. Role of C-terminal region of HA-33 component of botulinum toxin in hemagglutination. *Biochem. Biophys. Res. Commun.* 288, 650–657.

Schiavo, G., Matteoli, M., and Montecucco, C. 2000. Neurotoxins affecting neuro-exocytosis. *Physiol. Rev.* 80, 717–766.

Shapiro, R.L., Hatheway, C., and Swedlow, D.L. 1998. Botulism in the United States: A clinical and epidemiologic review. *Annals Internal Medicine* 129, 221–228.

Sharma, S.K., and Singh, B.R. 2004. Enhancement of the endopeptidase activity of purified botulinum neurotoxins A and E by an isolated component of the native neurotoxin associated proteins. *Biochemistry* 16, 4791–4798.

Shoham, D. 2000. Iraq's biological warfare agents: A comprehensive analysis. *Crit. Rev. Micro.* 26, 179–204.

Shotgun sequence of *C. botulinum* type Hall Strain A (ATCC 3502).http://www.sanger.ac.uk/Projects/C_botulinum/.

Shukla, H.D., Sharma, S.K., and Singh, B.R. 1997. Identification of a hemagglutinin present in the neurotoxin complex of *Clostridium botulinum* type A, as a heat shock protein. *Abstract K-127 of the 97thASM General Meeting*. May 4–8; Miami Beach, Florida.

Smart, J.K. 1997. History of chemical and biological warfare: An American perspective, in *Medical Aspects of Chemical and Biological Warfare*: Office of the Surgeon General, *Textbook of Military Medicine*; part I, Sidell, F.R. and Takafuji, E.T., Eds., Washington DC, 9–86.

Swaminathan, S., and Eswaramoorthy, S. 2000. Structural analysis of the catalytic and binding sites of *Clostridium botulinum* neurotoxin B. *Nat. Structl. Biol.* 7, 693–699.

Torii, Y., Tokumaru, Y., Kawaguchi, S., Izumi, N., Maruyama, S., Mukamoto, M., Kozaki, S., and Takahashi, M. 2002. Production and immunogenic efficacy of botulinum tetravalent (A, B, E, F) toxoid. *Vaccine* 20, 2556–2561.

Tucker, J.B. 2000. Toxic terror. In *Assessing the Terrorist Use of Chemical and Biological Weapons*, ed. J.B. Tuckr, 30–51. Cambridge, MA: MIT Press.

Turton, K., Chaddock, J.A., and Acharya, K.R. 2002. Botulinum and tetanus neurotoxins: Structure, function and therapeutic utility. *Trends Biochem. Sci.* 27, 552–558.

Woodruff, B.A., Griffin, P.M., McCroskey, L.M., Smart, J.F., Wainwright, R.B., Bryant, R.G., Hutwagner, L.C., and Hatheway, C.L. 1992. Clinical and laboratory comparison of botulism from toxin types A, B, and E in the United States, 1975–1988. *Journal of Infectious Diseases* 166, 1281–1286.

Woodward, L.A., Arimitsu, H., Hirst, R., and Oguma, K. 2003. Expression of HC subunits from *clostridium botulinum* types C and D and their evaluation as candidate vaccine antigens in mice. *Infect. Immun.* 71, 2941–2944.

Yongtai, Z., Sugiyama, H., Nakano, H., and Johnson, E.A. 1995. The Genes for the *Clostridium botulinum* Type G Toxin Complex Are on a Plasmid. *Infection Immunity* 63, 2087–2091.

Yowler, B.C., Kensinger, R.D., and Schengrund, C.L. 2002. Botulinum Neurotoxin A activity is dependent upon the presence specific gangliosides in neuroblastoma cells expressing synaptotagmin I. *J. Biol. Chem.* 277,32815–32819.