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Immunoprecipitation of Native Botulinum Neurotoxin Complexes from *Clostridium botulinum* Subtype A Strains

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Botulinum neurotoxins (BoNTs) naturally exist as components of protein complexes containing nontoxic proteins. The nontoxic proteins impart stability of BoNTs in the gastrointestinal tract and during purification and handling. The two primary neurotoxin complexes (TCs) are (i) TC1, consisting of BoNT, nontoxin-nonhemagglutinin (NTNH), and hemagglutinins (HAs), and (ii) TC2, consisting of BoNT and NTNH (and possibly OrfX proteins). In this study, BoNT/A subtypes A1, A2, A3, and A5 were examined for the compositions of their TCs in culture extracts using immunoprecipitation (IP). IP analyses showed that BoNT/A1 and BoNT/A5 form TC1s, while BoNT/A2 and BoNT/A3 form TC2s. A *Clostridium botulinum* host strain expressing recombinant BoNT/A4 (normally present as a TC2) from an extrachromosomal plasmid formed a TC1 with complexing proteins from the host strain, indicating that the HAs and NTNH encoded on the chromosome associated with the plasmid-encoded BoNT/A4. Strain NCTC 2916 (A1/silent B1), which carries both an *ha* silent *bont/b* cluster and an *orfX bont/a1* cluster, was also examined. IP analysis revealed that NCTC 2916 formed only a TC2 containing BoNT/A1 and its associated NTNH. No association between BoNT/A1 and the nontoxic proteins from the silent *bont/b* cluster was detected, although the HAs were expressed as determined by Western blotting analysis. Additionally, NTNH and HAs from the silent *bont/b* cluster did not form a complex in NCTC 2916. The stabilities of the two types of TC differed at various pHs and with addition of KCl and NaCl. TC1 complexes were more stable than TC2 complexes. Mouse serum stabilized TC2, while TC1 was unaffected.

Botulinum neurotoxins (BoNTs) are produced by anaerobic, Gram-positive, spore-forming bacteria, including *Clostridium botulinum* and rare strains of *Clostridium baratii* and *Clostridium butyricum*. BoNTs are the most poisonous protein toxins known in nature, and they are classified as tier 1 category A select agents due to their potential use as bioterrorism agents (1). Purified BoNT is a protein with a molecular mass of ca. 150 kDa, consisting in its active form of a heavy chain (Hc) (~100 kDa) and a light chain (Lc) (~50 kDa) linked by a disulfide bond and noncovalent molecular interactions (2). The heavy chain contains two functional domains, a receptor binding domain and a translocation domain (3, 4), while the light chain is responsible for catalytic activity on SNARE substrates in the neuronal cell cytosol. BoNTs are distinguished immunologically into seven serotypes designated A to G based on toxin neutralization using homologous antitoxins (5). Recently, the existence of an eighth serotype, H, in a dual-toxin-producing strain of *C. botulinum* has been reported (5).

Of the seven established serotypes, BoNT/A is of particular interest since it is the most potent BoNT, exhibits longer duration in humans than other serotypes, and is also implicated in most cases of human botulism in the continental United States (6, 7). Within serotype A, five subtypes of BoNT/A (A1 to A5) have been identified by nucleotide sequence analyses (8–11), and their 150-kDa neurotoxins have been purified (8, 11–15). Recently, two additional *bont/a* genes have been reported from distinct dual-BoNT-producing strains. The nucleotide and deduced protein sequences of these two type A toxins differ marginally from the sequences of the subtype A1 to A5 toxin genes (16, 17). These new toxins may comprise subtypes BoNT/A6 and BoNT/A7, but their neurotoxins have not yet been purified for characterization.

It has been recognized since the 1940s that BoNTs naturally form protein complexes (18, 19), wherein BoNT associates with nontoxic protein components. Early attempts to isolate the BoNTs from *C. botulinum* cultures were made by acid precipitation of culture supernatant by adjusting the pH to 3.5 to 4 (sometimes aided by the addition of RNA), which resulted in concentration of toxicity in the precipitated material (20). Subsequent studies by ultracentrifugation (21) and diffusion (22) showed that highly toxic needle-shaped "paracrystals" of a 900-kDa complex were formed in type A cultures. Additional studies showed that BoNT constituted only a fraction of the 900-kDa complex (23). The association of nontoxic complexing proteins with neurotoxin was further demonstrated when the large 900-kDa complex exhibiting hemagglutinating activity was adsorbed by erythrocytes but did not affect the toxin titer (23). Additional studies of the BoNT complexes using ultracentrifugation at various pHs and ionic strengths showed slower sedimentation boundaries, supporting the presence of lower-molecular-mass components in the large 900-kDa complex. Conclusive evidence of the nature of the toxin complexes (TCs) was obtained when ion-exchange chromatographic methods became available. Chromatography of the BoNT crystal solution using DEAE-Sephadex with an NaCl gradient gave one peak, representing about 20% of the protein complex, with high toxicity and little hemagglutinating activity (23). The second peak had high hemagglutinating activity but low toxicity (24, 25).

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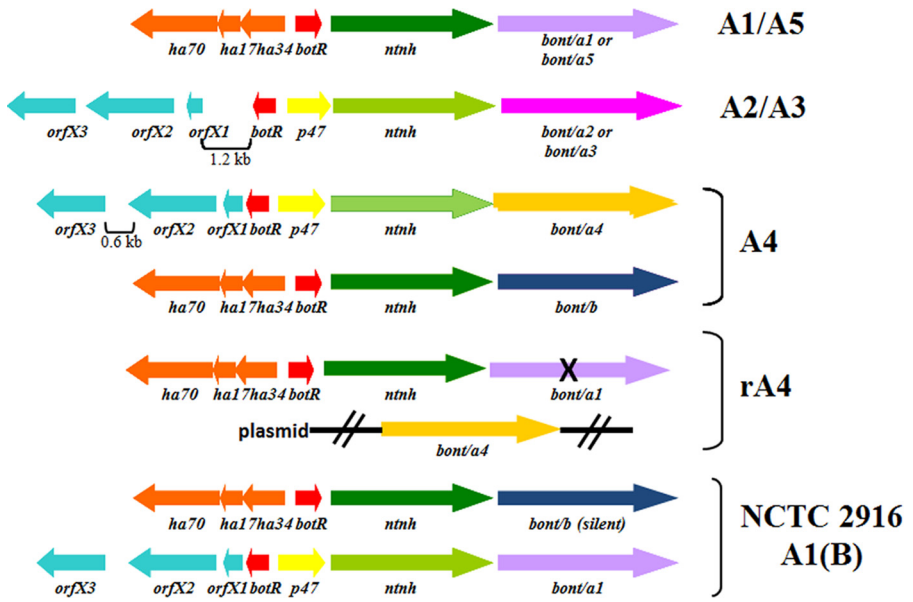


FIG 1 BoNT gene cluster arrangements of the *C. botulinum* type A strains used in this study.

These chromatography experiments definitively demonstrated that the BoNTs exist in complexes with nontoxic proteins in their natural state. Three main types of BoNT complexes have been identified, i.e., LL (~900 kDa), L (~500 kDa), and M (~300 kDa), differing in size based on quantities of BoNT and various nontoxic protein components (23). The LL complex is found only in certain type A strains, while L complexes have been demonstrated in serotypes A and in proteolytic strains of serotypes B and F. The M complexes are found in all serotypes and consist of only nontoxin-nonhemagglutinin (NTNH) and BoNT components.

The protein analyses of BoNT complexes have been complemented by gene sequencing approaches. Accordingly, sequence analyses of the BoNT genes and their flanking regions revealed that the genes encoding the nontoxic complex components are organized in gene clusters with the BoNT gene. The content and the arrangement of the genes in the toxin gene clusters differ depending on the BoNT serotype and subtype (26–29). Two types of toxin gene clusters have been identified: a hemagglutinin gene (*ha*) cluster and an *orfX* cluster (encoding proteins with unknown functions). The *ha* cluster consists of genes encoding hemagglutinin 17 (HA17), HA33, HA70, BotR, NTNH, and BoNT. The *orfX* cluster generally consists of genes encoding OrfX1, OrfX2, OrfX3, BotR, P47, NTNH, and BoNT. However, nonproteolytic strains of type E and some type F strains lack the *botR* gene. Only in serotype A strains could *bont* genes exist in either *ha* or *orfX* clusters.

As shown in previous reports, BoNT/A5 and the majority of BoNT/A1 genes are found in *ha* clusters, while BoNT/A2, BoNT/A3, and BoNT/A4 genes are in *orfX* clusters; however, in some strains, the A1 toxin gene is found in an *orfX* cluster (9, 29–34). Many strains that produce BoNT/A1 also carry a silent BoNT/B gene (e.g., NCTC 2916) and have both types of clusters, an *ha*-associated *bont/b* (silent) gene cluster and an *orfX*-associated *bont/a1* cluster. The diversity and variations of neurotoxin gene clusters (Fig. 1) suggest that horizontal gene transfer recombination has occurred during evolution (29, 35, 36).

Humans and animals can become infected with BoNT through three primary routes: ingestion of food containing *C. botulinum* toxin (foodborne botulism), *C. botulinum* colonization and BoNT formation in the intestinal tract (primarily in infants) (intestinal botulism), and BoNT produced by *C. botulinum* growing in wounds (wound botulism) (23). It has been demonstrated that BoNT complexes protect the toxin from harsh conditions, including pH, salt, temperature, and digestive enzymes, in the gut and may promote epithelial penetration (37–46).

Several studies have been performed to elucidate the biological and structural roles of the nontoxic botulinum complex proteins. Most of the studies have been performed on toxin complexes purified by chromatography or by *in vitro* reconstitution with recombinant BoNTs and nontoxic complex protein components expressed in *Escherichia coli* (45, 47–49). Structural studies of TCs have mainly used electron microscopy (EM), since high-quality crystals for X-ray diffraction of the LL and other complexes have not been obtained from native cultures. The EM studies have shown different lobes believed to correspond to the BoNT and complexing proteins (49–51).

The goal of this study was to evaluate the composition of the BoNT/A subtype complexes formed in native bacterial cultures. Specifically, we were interested in the composition of complexes before they were subjected to commonly used purification methods, especially chromatography and equilibration in buffers of various pHs. In this study, we used immunoprecipitation (IP), a well-established technique to analyze interactions among proteins. IP uses antibodies to bind and isolate specific proteins, which could be components of larger protein complexes. By targeting the known protein with a specific antibody, it is feasible to capture the entire protein complex from the solution, and individual proteins can then be identified by SDS-PAGE, Western blotting, or other protein identification methods (52–54). Interactions between proteins can be characterized as stable or transient, and the strength/weakness of the protein interactions can be evaluated. Classic examples of protein complexes analyzed by IP are hemoglobin and RNA polymerase (54–56).

TABLE 1 *C. botulinum* strains used in this study

Strain	Toxin type	*bont* cluster type	Toxin complex type
Hall A-*hyper*	A1	*ha*	BoNT/A1-NTNH-HAs
Kyoto F	A2	*orfX*	BoNT/A2-NTNH
CDC A3	A3	*orfX*	BoNT/A3-NTNH
Hall A-*hyper*/tox$^-$(pMTL82152-*bont*/A4)	rA4	Originally *orf*, recombinant with *ha*	rBoNT/A4-NTNH-HAs
A661222	A5	*ha*	BoNT/A5-NTNH-HAs
NCTC 2916	A1/(silent B)	*orfX* with *bont/a1* and *ha* with *bont/b* (silent)	BoNT/A1-NTNH

In this study, we describe IP analyses of toxin complexes from culture extracts of *C. botulinum* strains producing BoNT/A subtypes 1 to 5. This minimally disruptive method was used to evaluate the composition and stability of the BoNT complexes. We have also found IP to be a sensitive method for analysis of BoNT production in cultures grown under different environmental and nutritional conditions.

MATERIALS AND METHODS

Biosafety and biosecurity. Our laboratory and personnel are registered with the CDC Select Agent Program for research involving botulinum neurotoxins (BoNTs) and BoNT-producing strains of clostridia. The research program, procedures, occupational health plan, documentation, security, and facilities are closely monitored by the University of Wisconsin—Madison Biosecurity Task Force, the University of Wisconsin—Madison Office of Biological Safety, the University of Wisconsin Select Agent Program, and the CDC and the Animal and Plant Health Inspection Service (APHIS) as part of the University of Wisconsin—Madison Select Agent Program. All personnel have undergone suitability assessments and completed rigorous and continuing biosafety training, including biosafety level 3 (BSL3) and select agent practices, before participating in laboratory studies involving BoNTs and neurotoxigenic *C. botulinum*. All animal experiments have been approved by the University of Wisconsin IACUC.

Strains, media, growth conditions, and toxin complex (TC) isolation. *C. botulinum* strains used in this study are listed in Table 1. *C. botulinum* strains were grown under anaerobic conditions for 96 h in 1.5 or 10 liters of toxin production medium (TPM), comprised of 2% NZ Case TT, 1% yeast extract, and 0.5% glucose (pH 7.4) for strains producing subtypes A1, A2, recombinant A4 (rA4), and A5; the A3 toxin-producing strain was grown in modified Mueller-Miller medium (13). NZ Case TT was from Kerry Biosciences, and all the other medium ingredients were from Difco. All salts were from Sigma-Aldrich.

Culture proteins were concentrated by acid precipitation as previously described (11–13, 57). Briefly, following incubation of *C. botulinum* for 96 h at 37°C, the cultures were cooled for 60 min on ice, and the pH of the total culture was gradually lowered to 3.5 by slow addition of 1.5 M H_2SO_4 with gentle stirring. The precipitate was collected by centrifugation and washed with distilled water. The pellet was extracted twice in 200 ml of 0.1 M sodium citrate buffer (pH 5.5) with gentle stirring for 2 h at room temperature. The extracts were centrifuged at 15,000 × *g* at 20°C for 15 min. Supernatants from the two extractions were combined, and proteins were precipitated by addition of solid ammonium sulfate to 60% saturation (39 g/100 ml) and kept at 4°C overnight.

IP analyses. Fifty microliters of Dynabeads-protein A (monosized, superparamagnetic Dynabeads coupled with protein A) was used as a carrier in the immunoprecipitation (IP) analyses. For preparation of the beads for IP analysis, 10 μg of antibodies (Abs) was incubated with the beads for 1 h at 25°C to allow the Fc regions of the Abs to bind to protein A coupled to the beads as described in the Dynabeads-protein A protocol. The antibodies used were polyclonal Abs to BoNT/A1 or BoNT/A2 or a monoclonal antibody (MAb) to HA50. The beads containing bound Abs were washed once with washing buffer provided in the kit to remove unbound Abs and used for the IP experiments. Culture extracts (0.5 to 1 mg total protein) in 1 ml were added to the bead-bound Abs and incubated for 1 h, followed by three washes with 50 mM Na citrate (pH 5.5) buffer. After washing, the beads were then boiled with 30 μl of elution buffer (provided in the kit) and 10 μl NuPAGE lithium dodecyl sulfate (LDS) sample buffer. DynaMag magnets purchased from Life Technologies were used to separate the beads from the supernatant, and the eluted sample was transferred to a clean tube to be further examined by SDS-PAGE.

SDS-PAGE analysis. Fifteen microliters of recovered protein eluted from the beads was mixed with 5 μl of NuPAGE LDS sample buffer (4×) and loaded on a NuPage 4 to 12% bis-Tris SDS-polyacrylamide gel using NuPAGE morpholineethanesulfonic acid (MES)-SDS running buffer (purchased from Life Technologies). Each sample was analyzed under nonreduced (no dithiothreitol [DTT] added) and reduced (with 100 mM DTT) conditions. SDS-polyacrylamide gels were run at 110 V for 1.5 h. The SeeBlue Plus2 prestained protein standard (Life Technologies) was used to estimate the molecular masses of protein bands.

Detection of BoNT complexes in culture extracts by IP. One milliliter of ammonium sulfate-precipitated culture extract containing 0.5 mg to 1 mg total protein from each *C. botulinum* A strain examined was collected by centrifugation at 16,100 × *g* for 5 min at room temperature and resuspended in 0.5 to 1 ml 20 mM NaH_2PO_4 buffer pH 6.0, in which toxin complexes (TCs) are stable. IP and SDS-PAGE analyses were performed as described above to determine the proteins in the TCs. IP was also performed on samples containing only crude extract without added antibody and on beads containing only antibody without added crude extract as negative controls.

Analysis of TC composition under various conditions by IP. Na_2HPO_4 (20 mM)-KH_2PO_4 (2 mM) solutions at different pH values (pH 4.5, 7.5, and 8.5) with or without 2.7 mM KCl and 137 mM NaCl were used to evaluate TC stability. Sera collected from mice (female ICR from Harlan Laboratories) were also used to evaluate TC stability. NaH_2PO_4 (20 mM, pH 6.0) was used as the standard buffer to dissolve ammonium sulfate-precipitated material from crude extracts. BoNT subtypes A1 and A2 were initially chosen for evaluation, as they represent the archetypal TC1 and TC2 produced by *C. botulinum* type A. Two approaches were used to study stability of the TCs. In the first approach, the stability of TCs was assessed by dissolving the precipitated extract of subtype A1 in 20 mM NaH_2PO_4 (pH 6.0) buffer. This extract was incubated with beads containing the capture antibody for 1 h and then washed with 50 mM Na citrate (pH 5.5)–20 mM sodium phosphate buffer at pH 7.5 or pH 8.5, with or without 2.7 mM KCl and 137 mM NaCl, to determine which proteins remained associated after elution from the beads. In the second approach, the crude extracts from *C. botulinum* subtype A1 or A2 were dissolved in different buffers, including phosphate buffer at pH 6.0, pH 7.5, or pH 8.5 with or without 2.7 mM KCl and 137 mM NaCl, followed by incubation for 60 min with beads containing capture antibody to see which proteins remained associated under these conditions. In this approach, the samples following incubation with the beads were washed with 50 mM Na citrate (pH 5.5) prior to elution. In addition, 500 μl mouse serum or 500 μl 20 mM phosphate buffer (pH 4.5) was used to dissolve the toxin extract before IP and incubated with antibodies for 1 h to observe the effects of serum and low pH on the stability of the complex. SDS-PAGE analyses

were performed as described above to assess the components of the IP-isolated complexes.

Evaluation of BoNT TCs in *C. botulinum* strain Hall A-*hyper*/tox$^-$(pMTL82152-*bont*/A4) expressing recombinant BoNT/A4 and in *C. botulinum* strain NCTC 2916 expressing BoNT/A1 in an OrfX cluster. BoNT/A4 is produced as a minor toxin and BoNT/B1 as the major toxin in the dual-toxin-producing *C. botulinum* strain 657Ba. Due to technical difficulties (15) in quantitating the minor toxin in dual-toxin-producing strains, BoNT/A4 titers have been estimated only indirectly by neutralization of BoNT/B to estimate remaining toxicity of BoNT/A4 by mouse bioassay. The ratio of BoNT/B to BoNT/A4 toxicity has been roughly estimated to be between 10:1 and 100:1 (58, 59). Thus, the recombinant BoNTs/A4 was constructed. Briefly, the gene encoding BoNT/A4 was amplified by PCR using genomic DNA isolated from strain 657Ba as a template, and the gene encoding recombinant *bont/a4* was inserted into a modular clostridial expression vector. The recombinant BoNT/A4 expression construct was transferred to a nontoxigenic *C. botulinum* expression strain that carries a chromosomally encoded HA-type toxin gene cluster with the toxin gene insertionally inactivated. As described above, in the native strain (657Ba), the *bont/a4* gene is present in an *orfX* cluster.

We also examined *C. botulinum* strain NCTC 2916, which produces BoNT/A1; however, this strain also contains a silent *bont/b* gene cluster. BoNT/B1 is not expressed due to a premature stop codon in the coding region of the gene (60). The *bont/a1* gene in this strain is arranged in an *orfX* gene cluster instead of an *ha* gene as cluster typically found in the majority of A1 strains, while the silent *bont/b* gene is located within an *ha* gene cluster. IP was performed on crude extracts of these two cultures by using 10 μg of anti-BoNT/A1 and -BoNT/A2 polyclonal Abs or anti-HA50 MAb to study whether the formation of chimeric neurotoxin complexes may occur.

Western blot analysis of HA expression in NCTC 2916. The expression of HAs in *C. botulinum* strain NCTC 2916 was verified by Western blotting using the WesternBreeze chemiluminescent Western blot immunodetection system purchased from Life Technologies. Anti-HA17, -HA20, and -HA50 MAbs were kindly provided by Larry Stanker. Anti-HA33 polyclonal Abs were purchased from Meridian Life Science.

RESULTS

Analysis of TCs by IP from *C. botulinum* BoNT/A1, BoNT/A2, BoNT/A3, and BoNT/A5. To study the composition of TCs in *C. botulinum* A subtype-producing strains, IP analyses were performed using culture extracts without additional purification by chromatography or other potentially more intrusive methods. TCs from *C. botulinum* strains producing subtypes A1, A2, A3, and A5 were initially examined. IP analyses showed that anti-BoNT/A1 or anti-BoNT/A2 Abs pulled down their expected TCs. Thus, the TC pulled down by IP from *C. botulinum* strain Hall A-*hyper* contained BoNT/A1, full-length NTNH, truncated NTNH (~120 kDa), HA50, HA33, HA20, and HA17 (Fig. 2A). Similar results were observed in culture extracts of *C. botulinum* strain A661222, which produces BoNT/A5 (Fig. 2D). TCs from strains Kyoto F (BoNT/A2) and CDC A3 (BoNT/A3) contained only BoNT and NTNH, without any OrfX proteins being detected (Fig. 2B and C). Negative controls (beads without conjugated antibodies with extracts and beads with conjugated antibodies in the absence of extracts) in each set of experiments showed that nonspecific binding between beads and culture extracts did not occur. TCs were eluted only together with the Abs.

Effects of pH and salts on TC stability. *C. botulinum* subtypes A1 and A2, which are typical of TC1 and TC2, were evaluated for stability under various pH and salt conditions. Two approaches were used to investigate stability (see Materials and Methods for details). BoNT/A1 TC was incubated with Ab-bound beads, and the beads were then washed at pH 5.5, 7.5, or 8.5 with or without 2.7 mM KCl and 137 mM NaCl. TCs were stable under these conditions, as similar quantities of TC proteins pulled down by IP were visualized by SDS-PAGE. Samples washed with 50 mM Na citrate (pH 5.5) were used as a control, since TC is stable under this condition (Fig. 3A). To further investigate stability of TCs of BoNT/A1 and/A2, protein extracts were resuspended in buffers at pHs 6.0, 7.5, and 8.5 with or without 2.7 mM KCl and 137 mM NaCl prior to IP analyses. BoNT/A1 TC proteins remained intact under the pH and salt conditions tested, as shown by SDS-PAGE (Fig. 3B). To investigate if other proteins from the extracts besides those present in TC1 could affect the stability of the TC, chromatography-purified A1 TC from our laboratory was also analyzed. The chromatography-purified TC1 was dissolved in buffers with different pHs and salts (as described above) and incubated for 1 h and 4 h (Fig. 3E and F). The analyses confirmed that A1 TC was stable under these conditions. Figure 3C shows that the composition and quantities of BoNT/A2 TC proteins (BoNT/A2 and NTNH) eluted under different pH and salt conditions remained approximately the same, indicating that the binding between BoNT/A2 and NTNH was stable. However, incubation at higher pHs (7.5 and 8.5) and in the presence of salts affected the composition of the TC2 complex, since reduced quantities of BoNT/A2 TC were captured by IP at pH 7.5 or 8.5 with or without salts compared to quantities at pH 6.0. Unexpectedly, the presence of salts increased the quantities of TC isolated by IP at the higher pHs.

We also evaluated whether BoNT/A1 TC1 and BoNT/A2 TC2 remained intact in mouse serum or at pH 4.5, as might be encountered in the human gut. Twenty millimolar sodium phosphate buffer at pH 4.5 or mouse serum was used to dissolve the precipitated protein extracts prior to IP analysis. Figure 3D shows that BoNT/A1 TC1 was stable at low pH and in mouse serum. On the other hand, reduced quantities of BoNT/A2 TC2 compared to BoNT/A1 TC1 were detected by IP at pH 4.5. In contrast, higher quantities of BoNT/A2 TC2 were detected by IP in mouse serum, indicating stability of BoNT/A2 TC proteins in serum.

Analysis of neurotoxin complexes formed in an rBoNT/A4 expression strain and in strain NCTC 2916. Two additional strains were evaluated to determine whether BoNTs produced in their native strains with one type of TC can form a complex with the nontoxic proteins from the other type of TC present in the bacterial genome. Recombinant BoNT/A4 was expressed in the nontoxigenic *C. botulinum* expression strain Hall A-*hyper*/tox$^-$ as previously described (15) This expression strain carries an *ha* cluster with its BoNT/A1 gene insertionally inactivated while still expressing all the nontoxic TC1 complex proteins (14) (Fig. 1). The *bont/a4* gene resides in an *orfX* cluster in its native strain 657Ba. The *bont/a4* gene alone without the *orfX* cluster genes was cloned into an expression vector and the vector conjugated into the expression strain. IP analysis of the protein extracts prepared from the rA4 expression strain showed that a TC1 containing rBoNT/A4, NTNH, HA17, HA20, HA33, and HA50 was formed (Fig. 4A). The results indicated that BoNT/A4 and nontoxic complex proteins formed a "hybrid" TC1 even when the BoNT/A4 and TC genes were differentially located on a plasmid and the chromosome, respectively.

NCTC 2916 carries two BoNT gene clusters, an *orfX* cluster with *bont/a1* and an *ha* cluster with a silent *bont/b*, but produces only active BoNT/A1. The two toxin gene clusters are located on

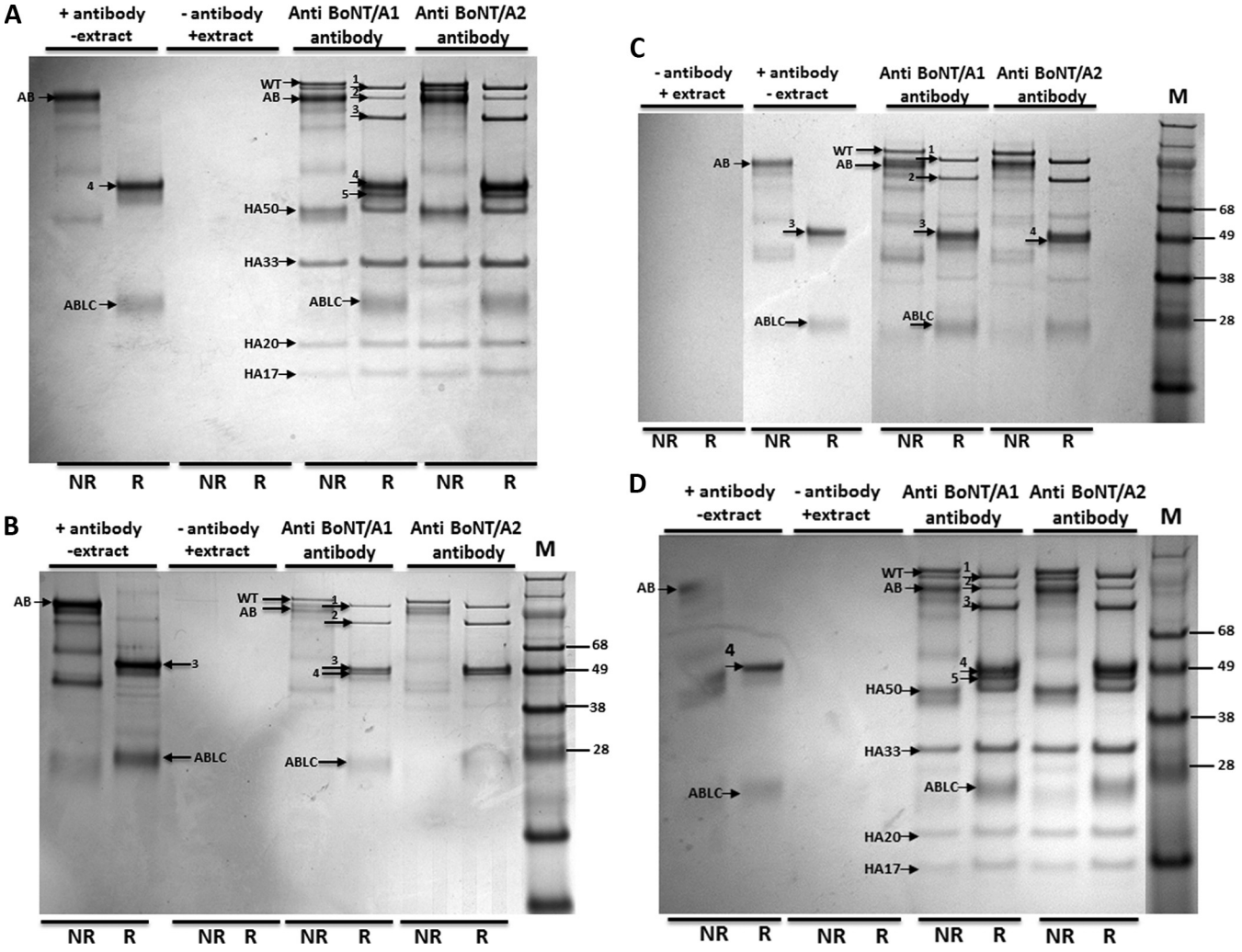
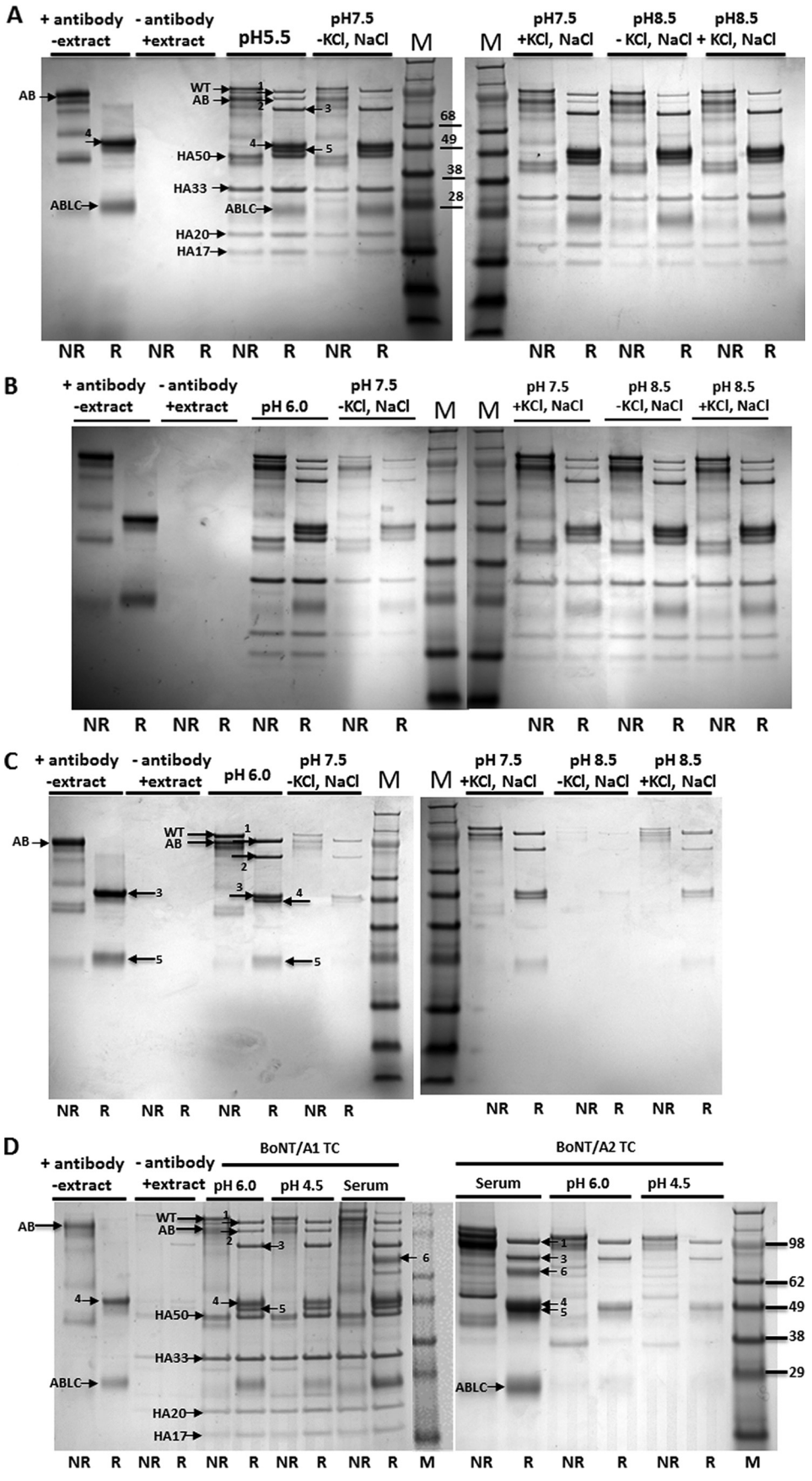


FIG 2 Coomassie blue-stained SDS-PAGE analyses of TCs pulled down by IP using anti-BoNT/A1 or anti-BoNT/A2 capture antibodies with *C. botulinum* subtypes A1 (A), A2 (B), A3 (C), and A5 (D). Abbreviations: NR, nonreduced samples; R, samples reduced with 100 mM DTT. (A and D) WT, 150-kDa holotoxin; AB, full-length Ab; ABLC, light chain of Ab; 1, NTNH; 2, truncated NTNH; 3, BoNT Hc; 4, heavy chain of Ab; 5, BoNT/A Lc; negative controls, beads with anti-BoNT/A1 Ab only without crude culture extract and beads with crude culture extract only without Ab. (B and C) WT, 150-kDa holotoxin; AB, full-length Ab; ABLC, light chain of Ab; 1, NTNH; 2, BoNT Hc; 3, heavy chain of Ab; 4, BoNT Lc; negative controls, beads with anti-BoNT/A2 Ab without crude culture extract and beads with crude culture extract without Ab.

the same chromosome but at distant locations. In strain NCTC 2916, IP analysis showed that a TC2 containing BoNT/A1 and NTNH from the *orfX* gene cluster was formed (Fig. 4B). TC1 containing BoNT/A1 from the *orfX* gene cluster and NTNH and HAs expressed from the silent *bont/b* gene cluster was not detected. Western blot analysis verified that all HA proteins, HA17, HA20, HA50, and a truncated HA33, were produced in the culture of NCTC 2916 even though BoNT/B was not expressed (Fig. 4C). These results indicate that when BoNT/A1 and nontoxic proteins of both TC1 and TC2 are produced in NCTC2916 (and possibly other strains with silent gene clusters), BoNT forms a TC with its original associated complex proteins. Furthermore, in order to understand whether the nontoxic proteins (NTNH and HAs) associated with the silent *bont/b* cluster may form a complex without a BoNT component in NCTC 2916, IP was performed using an anti-HA50 MAb. The results showed that a nontoxin complex was not formed. BoNT/A1 TC1 was detected from Hall A-*hyper* culture extract by IP with anti-HA50 MAb, indicating that the anti-HA50 MAb isolated a protein complex with HA50 as a component. (Fig. 4D).

DISCUSSION

Previous studies have shown that BoNT/A1 and BoNT/A5 form a TC1 comprised of BoNT/A, NTNH, and HAs (11). In contrast, BoNT/A2 and BoNT/A3 form a TC2 containing only BoNT/A and NTNH without OrfX proteins present (11–13). In previous analyses of BoNT complexes, TCs were isolated using several chromatography separation steps, with changes of buffers, dialysis, and other manipulations. Purification steps used in obtaining TC2 could potentially cause the loss of OrfX proteins present in cultures. In this study, we sought to determine if the method of TC isolation could affect their protein composition. The goal of this study was to determine the protein composition of the TCs in culture extracts with minimal manipulation, thus avoiding the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ABLC
WT
AB
HA50
HA33
HA20
HA17
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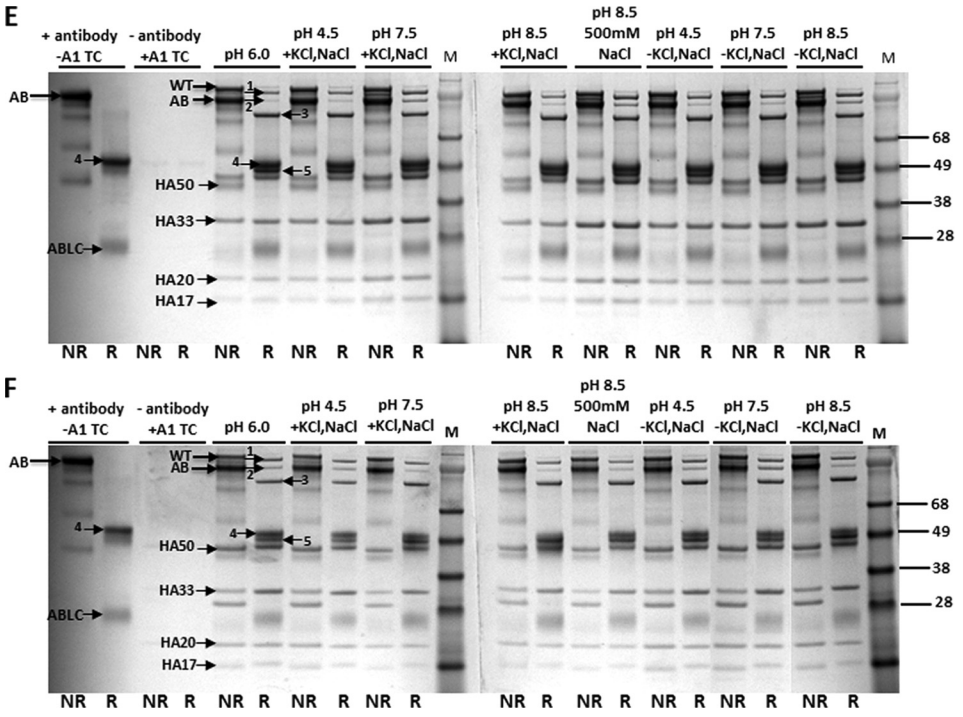

FIG 3 Coomassie blue-stained SDS-PAGE analyses of *C. botulinum* TCs of BoNT/A1 and BoNT/A2 pulled down by IP when BoNT extracts were exposed to different pHs and salts. (A) BoNT/A1 TC1 washed at different pHs with or without salts after IP; (B) BoNT/A1 extract dissolved at different pHs with or without salts prior to IP; (C) BoNT/A2 extract dissolved at different pHs with or without salts prior to IP; (D) BoNT/A1 or BoNT/A2 extract incubated at pH 4.5 or in mouse serum for 1 h prior to IP; (E and F) BoNT/A TCs (purified by chromatography) incubated at different pHs with or without salts for 1 h (E) or 4 h (F) prior to IP. All SDS-PAGE analyses were conducted using both nonreduced and reduced conditions as indicated. N, nonreduced samples; R, reduced samples. (A and B) Negative controls, beads with anti-BoNT/A1 without adding crude culture extract and beads with crude culture extract without Ab. (C) Negative controls, beads with anti-BoNT/A2 without adding crude toxin extract and beads with crude culture extract without Ab. (B, C, and D) Positive control, crude extract dissolved in 20 mM $NaPO_4$ (pH 6.0); TCs were washed with 50 mM Na citrate buffer (pH 5.5) after binding to Ab-bound beads. (A, D, E, and F) WT, 150-kDa holotoxin, AB, full-length Ab; ABLC, light chain of Ab; 1, NTNH; 2, truncated NTNH; 3, BoNT Hc; 4, heavy chain of Ab; 5, BoNT Lc; 6, serum protein (in panel D). (C) WT, 150-kDa holotoxin; AB, full-length Ab; 1, NTNH; 2, BoNT Hc; 3, heavy chain of Ab; 4, BoNT Lc; 5, light chain of Ab.

potential loss of complexing proteins due to purification methods. For this analysis, Abs to BoNTs and HA50 were used to precipitate TCs by IP from toxin extracts of native *C. botulinum* cultures. The *C. botulinum* strains evaluated included *C. botulinum* producing the subtypes BoNT/A1, BoNT/A2, BoNT/A3, and BoNT/A5, as well as a recombinant strain that produces BoNT/A4 and strain NCTC 2916, which contains genes expressing both TC1 and TC2 nontoxic complexing proteins.

IP analyses of culture extracts showed that BoNT/A1 and BoNT/A5 formed a TC1 with NTNH, HA17, HA20, HA50, and HA33, which is consistent with previous reports of TCs purified by precipitation and chromatographic procedures (11, 23). The IP analyses also showed that BoNT/A2 and BoNT/A3 formed a TC2 with NTNH alone but not containing OrfX proteins. In previous studies, we reported that TCs of subtypes A2 and A3 consist only of toxin and NTNH after going through chromatographic purification steps, even though OrfX proteins, (with the exception of OrfX3) were detected in the cultures using Western analyses (12). There has been discussion that the methods previously used for purification of TC2s resulted in the loss of OrfX proteins in TC2 due to weaker binding between OrfX proteins and BoNT and NTNH. The results of this study using IP and of previous studies describing the components of TC2s after precipitation and chromatographic purification (12, 13) support the conclusion that BoNT/A2 and BoNT/A3 TCs consist only of BoNT and NTNH.

In addition to strains producing the BoNT/A subtypes BoNT/A1, BoNT/A2, BoNT/A3, and BoNT/A5, two other strains were analyzed by IP. A rBoNT/A4-producing strain with the *bont/a4* gene expressed from a plasmid and the nontoxic complexing proteins produced from the genes located on the chromosome in the host strain was analyzed. NCTC 2916, which contains both an *ha*-associated silent *bont/b* cluster and an OrfX-associated *bont/a1* cluster, was also evaluated. IP analyses showed that rBoNT/A4 formed a full-complement TC1 (Fig. 4A). Furthermore, the detection of the "hybrid" TC1 in this recombinant strain indicates that it is not necessary for the genes encoding BoNT and nontoxic proteins to be present in the same gene cluster or same chromosomal locations, since the nontoxic gene cluster components *ha* and *ntnh* were expressed from the chromosome and *bont/a4* was expressed from a plasmid. These results are analogous to those with certain other protein complexes in bacteria, strikingly illustrated by the ribosome, where proteins and RNA from multiple chromosomal locations are able to combine and form functional complexes. Our results show that distal BoNT complex proteins are able to assemble in the confines of the cytoplasm or during secretion.

To further investigate the formation of "hybrid" TCs, strain NCTC 2916, which contains two distinct toxin gene clusters, was examined. The IP analyses of strain NCTC 2916 showed that the two sets of nontoxic proteins produced from the *ha*-associated

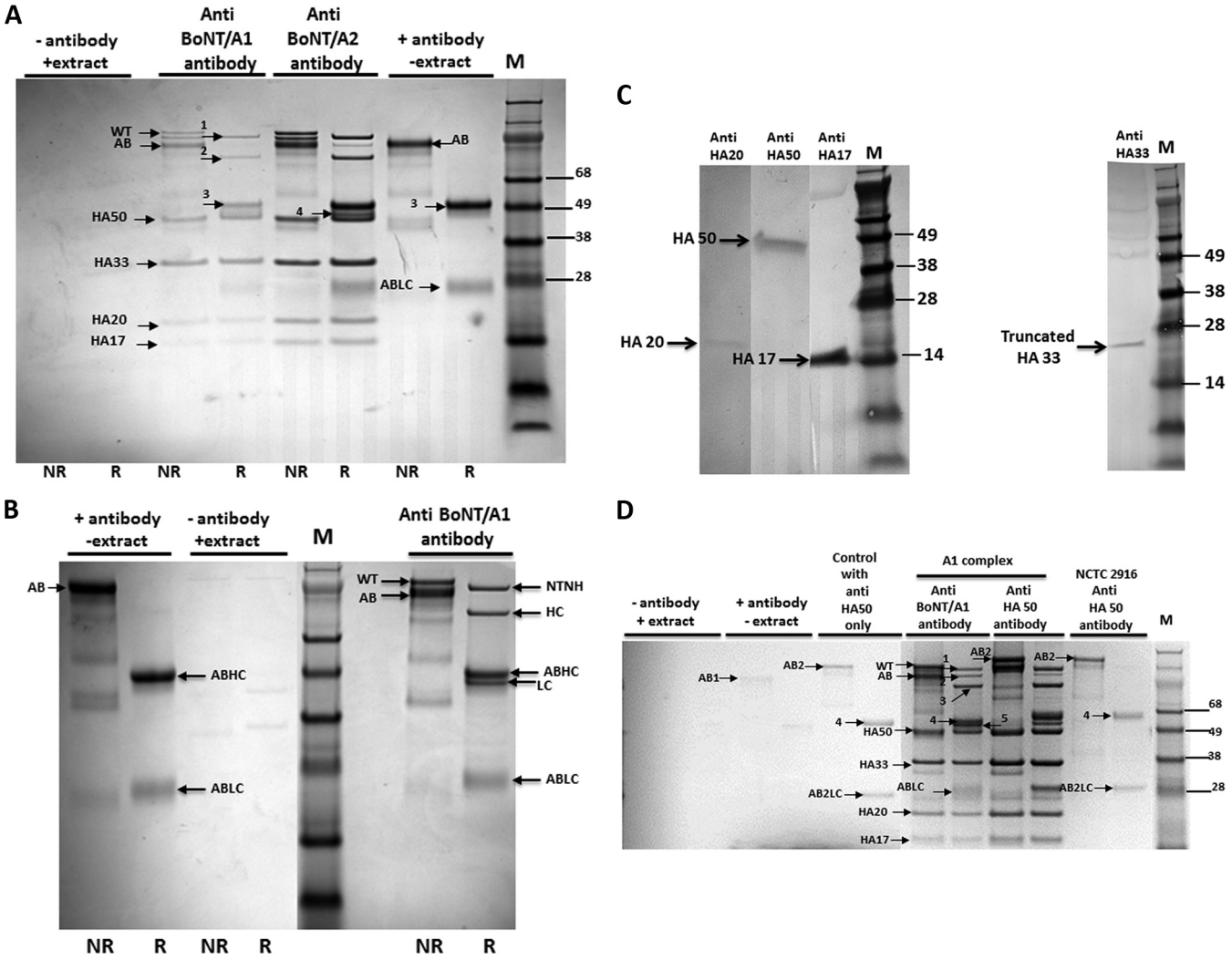

FIG 4 Analysis of "hybrid" TCs in an rBoNT/A4 expression strain and in strain NCTC 2916 (A1/silent B) using IP and Western blotting. (A) Coomassie blue-stained SDS-PAGE analyses of rBoNT/A4 TC pulled down by IP from culture extract using anti-BoNT/A1 or anti BoNT/A2 Abs. NR, nonreduced samples; R, reduced samples; WT, 150-kDa holotoxin; AB, full-length Ab; ABLC, light chain of Ab; 1, NTNH; 2, BoNT Hc; 3, heavy chain of Ab; 4, BoNT Lc; negative control, beads with anti-BoNT/A2 without added crude extract and beads with crude extract without Ab. (B) Coomassie blue-stained SDS-PAGE analyses of BoNT/A1 TC in NCTC 2916 pulled down by IP using anti-BoNT/A1 Ab. WT, 150-kDa holotoxin; AB, full-length Ab; HC, BoNT Hc; LC, BoNT Lc; ABHC, heavy chain of Ab; ABLC, light chain of Ab. (C) Western blot analysis of expressed HA proteins in strain NCTC 2916 with MAbs against HA17, HA20, or HA50 and polyclonal Abs against HA33. (D) Coomassie blue-stained SDS-PAGE analyses of TCs pulled down by IP in NCTC 2916 using an anti-HA50 MAb. Subtype A1 crude extract analyzed with IP using anti-HA50 MAb was used as positive control. WT, 150-kDa holotoxin; AB1, full-length anti-BoNT/A1 polyclonal Abs; AB2, full-length anti-HA50 MAb; ABLC, light chain of anti-BoNT/A1 polyclonal Abs; AB2LC, light chain of HA50 MAb; 1, NTNH; 2, truncated NTNH; 3, BoNT Hc; 4, heavy chain of MAb; 5, BoNT Lc.

cluster and the *orfX*-associated cluster in the culture, with only BoNT/A1 functionally expressed, the BoNT formed a TC only with its original associated cluster proteins but not with proteins expressed from the distal gene cluster. The difference between TC formation in *C. botulinum* rBoNT/A4 and NCTC 2916 is unclear. A possible explanation is that in NCTC 2916 the BoNTs and complex proteins are transcribed and translated at the same time as a transcriptional unit (61), thus preventing TC assembly with complex proteins from the distal BoNT gene cluster. Several other explanations are plausible, including protein degradation in the presence of an unexpressed or truncated BoNT or the need for coexpressed escort and chaperones for assembly. Western blot analysis of NCTC 2916 culture extract revealed a truncated HA33 protein. HA33 has been reported to be protease resistant and to stabilize and protect BoNT/A1 (42, 62, 63). Future studies are needed to better understand the relationship of the nontoxic complex proteins in multi-BoNT-producing strains and in strains containing a silent unexpressed BoNT.

The stability of TCs in buffers with and without salts at different pHs and in mouse serum was also studied. In this study, the proteins in the TC1 complex were stable as a complex under all conditions tested, including pHs from 4.5 to 8.5. These results appear to differ from previous reports (45, 64) where TCs were shown to be partly dissociated at pH 6.0 and fully dissociated at pH 7.6 based on chromatographic analyses. As one explanation, TCs evaluated by IP were not subjected to the same forces encoun-

tered by TCs prepared by chromatographic methods, buffer incubations, salt gradients, and other steps. Conformational changes of BoNTs captured by Abs could also impact the stability of TCs.

TC1 remained intact and stable at various pHs, in the presence of salts, and in mouse serum. In contrast, less TC2 was recovered under certain experimental conditions. The quantity of TC2 detected by IP was affected by pH and salts. Increasing pHs resulted in lower quantities of BoNT/A2 TC2 detected compared with the quantity detected at pH 6.0. The presence of salts increased the quantity of TC2 bound to the antibody detected by IP at pH 7.5 and pH 8.5, but the quantities detected were still less than at pH 6.0. The differences seen in IP results between BoNT/A1 TC and BoNT/A2 TC at various pHs may be enhanced by conformational interactions with the nontoxic complex proteins. When incubated in mouse serum, BoNT/A1 TC1 and BoNT/A2 TC2 were detected at similar levels, possibly due to the presence of stabilizing serum proteins. We plan to test the oral toxicity of purified TC1 and TC2 in a mouse model to further explore the role that complexing proteins play in the intoxication process.

This study raises questions on the function that nontoxic complexing proteins provide in stabilizing and protecting BoNTs and effects on oral intoxication. Nakamura et al. (44) postulated that HAs are involved in binding to intestinal acceptors and facilitating toxin absorption. TC2s appear to consist only of NTNH and BoNT, suggesting that NTNH may perform a function similar to that of complex proteins that have a role in intestinal absorption. Lastly, the ability of BoNT/A4 expressed from a plasmid to form a TC1 with NTNH and HAs expressed from the chromosome in the recombinant BoNT/A4 expression strain brings into question the intricate process of complex assembly and secretion, potentially guided by posttranslational events involving chaperones, other proteins, or RNA.

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