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Letters to the Editor

Further Characterization of Proteolytic *Clostridium botulinum* Type A5 Reveals that Neurotoxin Formation Is Unaffected by Loss of the *cntR* (*botR*) Promoter Sigma Factor Binding Site▽

Although widely recognized as forming a deadly food-borne intoxicant, proteolytic *Clostridium botulinum* also infects humans, causing infant botulism after growth and neurotoxin formation in the gut (1) and wound botulism after contamination of a wound, often following injected drug abuse (8). The genes encoding the neurotoxin and its accessory proteins form two operons whose expression is induced by CntR (BotR), an alternative sigma factor (5). Comparative genomics of proteolytic *C. botulinum* recently identified four wound botulism strains as being closely related, each carrying identical copies of a new subtype (A5) of the A neurotoxin gene (3). These strains were isolated in 2004 from patients in four different United Kingdom cities. The close relationship between three of these strains has been confirmed by amplified fragment length polymorphism (AFLP) using the method of Brett et al. (2). In the present study, 22 isolates from wound botulism and two from food-borne botulism clustered into seven different AFLP types. Eleven isolates were AFLP type 1, including three of the four wound botulism strains (one was not tested). One of these strains, H04402 065, is further described here.

Pulsed-field gel electrophoresis (PFGE) and Southern analysis of strain H04402 065, performed according to the method of Franciosa et al. (6), showed that the neurotoxin gene cluster was chromosomally located, as with type A1 neurotoxin genes (10, 12), while some type A2 and all types A3 and A4 are plasmid borne (6, 12). DNA sequencing of this region revealed a truncated, nonfunctional type B3 gene (7) in the 3′ flank. Most interestingly, there was a 76-bp deletion in the intergenic region of *cntC* (*ha33*) and *cntR* (*botR*) (Fig. 1). Recently, a very similar arrangement in a type A5 infant botulism strain (IBCA94-0216) was reported by Dover et al. (4). The two type A5 neurotoxin clusters share 99.7% nucleotide sequence identity between the 3′ end of the *cntE* (*ha70*) gene and the 3′ end of the type B3 neurotoxin-encoding gene. Of the 41 nucleotide differences, 8 reside in the *cntE* (*ha70*) gene, and 29 are in the nonfunctional B3 neurotoxin-encoding gene. Importantly, we recognized that the 76-bp deletion specifically removed the −35 and −10 motifs situated upstream of *cntR* that had been identified as a putative CntR autoregulatory binding site (9). Of 38 other strains of proteolytic *C. botulinum* also possessing the *ha*$^+$ form of the neurotoxin gene cluster (8), selected as representing a wide range of diseases caused, geographical distribution and type of neurotoxin(s) formed (3), only the type B toxin-forming strain 2345 shared the same deletion, implying that this modification is quite rare. Dover et al. (4) suggested that the A5 neurotoxin gene, together with its *cntB* (*ntnh*) gene, may have been inserted into a type B neurotoxin cluster. Since microarray analysis showed that strain 2345 clustered with *Clostridium sporogenes* (3), it is tempting to postulate that a strain of *C. sporogenes* acquired a type B neurotoxin cluster by horizontal transfer to give a strain similar to strain 2345, which, following a further round of recombination at this locus, gave rise to the type A5 neurotoxin cluster now described.

Since type A5 strains lack what appears to be a key region of their *cntR* promoter, it was expected that the timing or extent of neurotoxin formation would be affected. To address this, neurotoxin formation was measured by enzyme-linked immunosorbent assay (ELISA) in duplicate batch cultures of the type A5 strain H04402 065 and the type A1 strain ATCC 3502. Surprisingly, no significant difference in neurotoxin formation was observed (Fig. 2), suggesting that the region is not essential for this function. How *cntR* expression is initiated and maintained in the absence of these binding motifs is not clear. The simplest option is for use of an adjacent but presently unidentified binding site. This implies that the alternative promoter region brought about by the deletion potentially

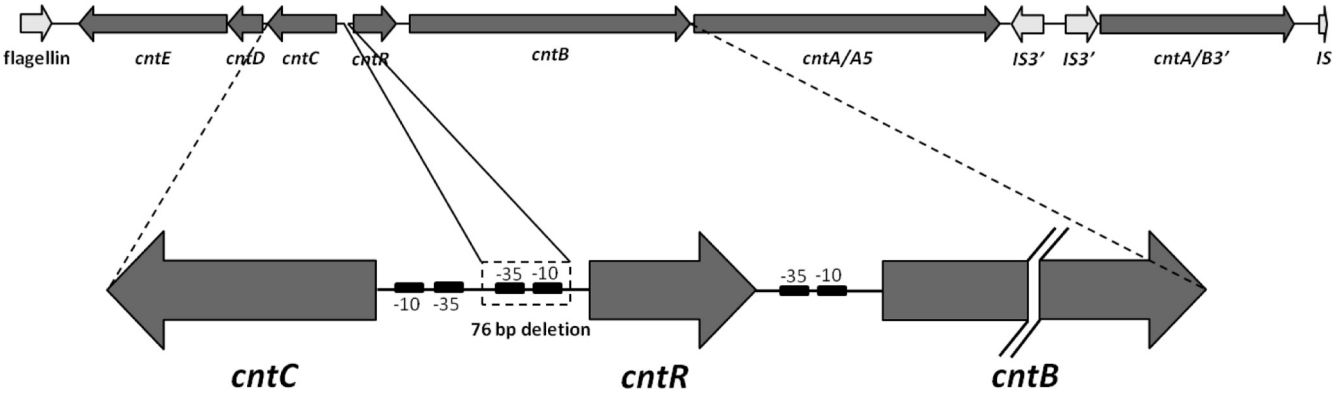

FIG. 1. Position of a 76-bp deletion identified in the neurotoxin gene cluster of proteolytic *C. botulinum* type A5 strain H04402 065. Neurotoxins and associated genes are shaded. Prime symbols denote a partial gene. Upper part of figure represents coding regions spanning 18.2 kb of a chromosomal DNA sequence (GenBank accession number GQ477161). The −35 and −10 binding sites for the CntR/RNA polymerase complex are indicated for each gene that is positively regulated by CntR. Alternative nomenclature for neurotoxin cluster genes is as follows: *cntC* (*ha34*), *cntD* (*ha17*), *cntE* (*ha70*), *cntR* (*botR*), *cntB* (*ntnh*), *cntA*/*A5* (*bont*/*A5*), *cntA*/*B3′* (*bont*/*B3′*).

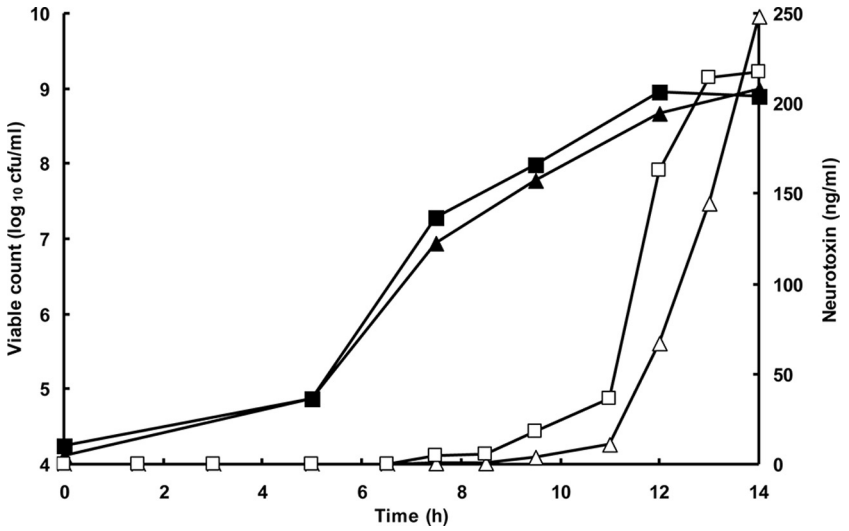

FIG. 2. Growth and neurotoxin formation by proteolytic *C. botulinum* type A5 strain H04402 065 (squares) and type A1 strain ATCC 3502 (triangles). Closed symbols represent the viable count (left *y* axis); open symbols represent the neurotoxin concentration in culture supernatant (right *y* axis).

coincides with the *cntC* gene promoter on the opposite DNA strand. This could cause transcriptional interference, a phenomenon in which overlapping promoters can cause one transcriptional unit to have a negative impact on its neighbor (11), one outcome of which might be reduced expression of the hemagglutinin-encoding operon. These hypotheses will be investigated by analysis of the transcriptome. The present observations suggest that the new type A5 strains may fortuitously offer the chance to dissect the control mechanisms of neurotoxin formation by proteolytic *C. botulinum*.

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