Evidence that Plasmid-Borne Botulinum Neurotoxin Type B Genes Are Widespread among *Clostridium botulinum* Serotype B Strains

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Abstract

**Background:** Plasmids that encode certain subtypes of the botulinum neurotoxin type B have recently been detected in some *Clostridium botulinum* strains. The objective of the present study was to investigate the frequency with which plasmid carriage of the botulinum neurotoxin type B gene (bont/B) occurs in strains of *C. botulinum* type B, Ab, and A(B), and whether plasmid carriage is bont/B subtype-related.

**Methodology/Principal Findings:** PCR-Restriction fragment length polymorphism was employed to identify subtypes of the bont/B gene. Pulsed-field gel electrophoresis and Southern blot hybridization with specific probes were performed to analyze the genomic location of the bont/B subtype genes. All five known bont/B subtype genes were detected among the strains; the most frequently detected subtype genes were bont/B1 and /B2. Surprisingly, the bont/B subtype gene was shown to be plasmid-borne in >50% of the total strains. The same bont/B subtype gene was associated with the chromosome in some strains, whereas it was associated with a plasmid in others. All five known bont/B subtype genes were in some cases found to reside on plasmids, though with varying frequency (e.g., most of the bont/B1 subtype genes were located on plasmids, whereas all but one of the bont/B2 subtypes were chromosomally-located). Three bivalent isolates carried both bont/A and /B genes on the same plasmid. The plasmids carrying the bont gene were five different sizes, ranging from ~55 kb to ~245 kb.

**Conclusions/Significance:** The unexpected finding of the widespread distribution of plasmids harboring the bont/B gene among *C. botulinum* serotype B strains provides a chance to examine their contribution to the dissemination of the bont genes among heterogeneous clostridia, with potential implications on issues related to pathogenesis and food safety.

Introduction

Botulinum neurotoxins (BoNTs) are zinc-dependent metalloproteases that inhibit the release of the neurotransmitter acetylcholine from peripheral cholinergic synapses. Through this mechanism of action, they can cause the flaccid paralysis of botulism in humans exposed through foodborne intoxication or intestinal and wound toxemias, thus representing a serious pathogenic and biodefense threat. However, when properly administered they can also provide therapeutic and cosmetic benefits in conditions characterized by excessive cholinergic activity [1].

Seven structurally similar but serologically distinct BoNT variants (A to G) have been defined: for example, BoNT/A has four distinct subtypes which differ from each other at the amino-acid level by up to 16% (BoNT/A1, /A2, /A3, and /A4), and BoNT/B has five subtypes, differing by up to 6% (BoNT/B1, /B2, /B3, non-proteolytic B, and bivalent B) [4].

All BoNT serotypes and subtypes are encoded by specific bont genes that may reside in the genome of six heterogeneous clostridia groups, comprising at least four distinct species (i.e., *Clostridium botulinum*, *C. argentinense*, *C. barati*, and *C. butyricum*) [2]. The bont/C and /D genes are located on distinct bacteriophages [5,6], and the bont/G gene is located on a plasmid [7,8], whereas the bont/A, /B, /E, and /F genes have long been assumed to be chromosomally located. The genome sequencing of the BoNT/A1-producing *C. botulinum* strain Hall (ATCC 3502) confirmed the chromosomal location of the bont/A1 subtype gene [9]. However, unexpectedly, the bont/A3 and bont/A4 subtype genes of the only two BoNT/A3- and /A4-producing strains identified to date (i.e., Loch Maree and 657) were recently shown to reside on large molecular weight plasmids (~267 kb and 270 kb, respectively)
[10]. Strain 657 forms greater amounts of bivalent BoNT/B than of BoNT/A4 and is thus regarded as C. botulinum type Ba [11]; its bivalent bont/B subtype gene was shown to reside on the same 270 kb plasmid that carries the bont/A4 subtype gene [10]. Nucleotide sequencing confirmed these results and also showed that the bont/B1 subtype gene of the C. botulinum strain Okra was located on a ~149 kb plasmid; the plasmids described in strains Loch Maree, 657, and Okra have been designated as “pCLK”, “p-CL”, and “pCLD”, respectively (GenBank Accession numbers CP000940 and CP000965) [12]. A ~48 kb plasmid (pCLL) from strain Ekland 17B (ATCC 25765) containing the non-proteolytic bont/A and pCLD subtype gene has been sequenced by the Los Alamos National Laboratory of the United States (GenBank Accession number CP001057), but has not been published to date.

These findings challenge the previous assumption of a chromosomal location of the bont/A and /B genes and raise questions as to whether plasmid-borne bont genes are rare or widespread among botulinum neurotoxicogenic clostridia.

BoNT/B is the most frequent cause of human botulism in Europe (including Italy) and the second leading cause of botulism in North America [13,14]. The purpose of the present study was to evaluate the genomic location of subtypes of the bont/B gene in a panel of C. botulinum strains of different origins. We also investigated the association between certain subtypes of the bont/A and B gene and plasmid carriage. The results are the first evidence that all five known bont/B subtype genes can reside on plasmids that vary considerably in size, and they clearly indicate that plasmid-borne bont/B genes are more widespread among C. botulinum serotype B isolates than previously known.

Results

PCR-Restriction Fragment Length Polymorphism (RFLP) subtyping of the bont/B gene

Based on the alignment of sequences of the bont/B gene (3876 bp) present in the GenBank database, the region encompassing nucleotides 920 to 3727 was selected for PCR amplification because it has shown a certain degree of nucleotide polymorphism. This region mainly encodes the transmembrane part of pulsed-field gels containing undigested genomic DNA from bacterial cells can be used to establish whether a certain gene is chromosomally or extra-chromosomally located [18,19]. The same strategy, based on pulsed-field gel electrophoresis (PFGE) and subsequent hybridization with bont/A and /B specific gene probes, has also been applied to show plasmid carriage of the bont/A3, /A4, and bivalent bont/B subtype genes [10]. We used this PFGE Southern blot approach to define the genomic location of the bont/B gene for the total 63 C. botulinum strains included in this study (Table 1); the genomic location of the bont/A gene in the 4 type Ab and 5 type A(B) C. botulinum strains was analyzed as well.

Three of the 63 C. botulinum strains (CDC-7827, displaying the bont/B1 PCR-RFLP subtype; ISS-310, displaying the bont/B2 PCR-RFLP subtype; and CDC-2312, displaying the bivalent bont/B PCR-RFLP subtype) repeatedly produced DNA smears after PFGE, presumably due to enhanced DNase activity; consequently, they were not suitable for the subsequent hybridization experiments, and the genomic location of their bont genes could not be determined by this technique.

The bont/B probe hybridized to the chromosomal band of 28 C. botulinum strains (47% of the total 60 PFGE-typeable strains tested). In the remaining 32 C. botulinum strains (53%), the bont/B probe hybridized to an extra-chromosomal band (Table 3). In particular, extra-chromosomal location was found for: 21/22 (95%) of the bont/B1 PCR-RFLP subtypes; 2/2 (100%) non-proteolytic bont/B subtypes; 7/13 (54%) bivalent bont/B subtypes, of which 6 atypically detected in non-bivalent C. botulinum strains (Table 1); 1/2 (50%) bont/B3 subtypes, the latter atypically detected in a

Genomic localization of the bont/B and /A subtype genes

Previous studies have demonstrated that Southern blot analysis of "bivalent strains" can be used to establish whether a certain gene is chromosomally or extra-chromosomally located [18,19]. The same strategy, based on pulsed-field gel electrophoresis (PFGE) and subsequent hybridization with bont/A and /B specific gene probes, has also been applied to show plasmid carriage of the bont/A3, /A4, and bivalent bont/B subtype genes [10]. We used this PFGE Southern blot approach to define the genomic location of the bont/B gene for the total 63 C. botulinum strains included in this study (Table 1); the genomic location of the bont/A gene in the 4 type Ab and 5 type A(B) C. botulinum strains was analyzed as well.

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Table 1. *C. botulinum* strains analyzed in this study, and genomic location of their bont/B and /A PCR-RFLP subtype genes.

| Strain no | Toxin type | State | Year | Source | bont/B subtype | bont/B genomic location | bont/A subtype | bont/A genomic location | plasmid size (kb) |
|-----------|------------|-------|------|--------|----------------|------------------------|----------------|------------------------|------------------|
| CDC-555   | B          | OH    | 1976 | corn   | B1            | plasmid                |                |                        | 245              |
| CDC-620   | B          | PA    | 1976 | IB     | B1            | plasmid                |                |                        | 245              |
| CDC-628   | B          | CT    | 1976 | uB     | B1            | plasmid                |                |                        | 217              |
| CDC-661   | B          | TN    | 1976 | IB     | B2            | chromosome             |                |                        |                  |
| CDC-668   | B          | TN    | 1976 | IB     | B1            | plasmid                |                |                        | 245              |
| CDC-706   | B          | AK    | 1976 | salmon | Bnp          | plasmid                |                |                        | 55               |
| CDC-816   | B          | MI    | 1977 | peppers | B3         | chromosome             |                |                        |                  |
| CDC-1588  | B          | AZ    | 1977 | IB     | Bbv          | plasmid                |                |                        | 170              |
| CDC-1632  | B          | PA    | 1977 | IB     | B1            | chromosome             |                |                        |                  |
| CDC-1758  | B          | OR    | 1977 | IB     | B1            | plasmid                |                |                        | 245              |
| CDC-1828  | B          | MD    | 1978 | IB     | B2            | chromosome             |                |                        |                  |
| CDC-1852  | B          | CO    | 1978 | FB     | Bbv          | plasmid                |                |                        | 245              |
| CDC-1872  | B          | MD    | 1978 | IB     | B1            | plasmid                |                |                        | 139              |
| CDC-2064  | B          | PA    | 1978 | IB     | B1            | plasmid                |                |                        | 139              |
| CDC-2094  | B          | MA    | 1978 | AB     | B1            | plasmid                |                |                        | 245              |
| CDC-2113  | B          | NJ    | 1978 | IB     | B1            | plasmid                |                |                        | 245              |
| CDC-2292  | B          | PA    | 1978 | IB     | B1            | plasmid                |                |                        | 245              |
| CDC-2306  | B          | NJ    | 1978 | IB     | B1            | plasmid                |                |                        | 245              |
| CDC-2312  | B          | PA    | 1978 | IB     | Bbv          | n.d. ⁶                 |                |                        |                  |
| CDC-2329  | B          | DE    | 1978 | IB     | B1            | plasmid                |                |                        | 245              |
| CDC-2358  | B          | MA    | 1978 | uB     | Bbv          | plasmid                |                |                        | 245              |
| CDC-2586  | B          | KY    | 1979 | IB     | B2            | chromosome             |                |                        |                  |
| CDC-2589  | B          | KY    | 1979 | FB     | B1            | plasmid                |                |                        | 139              |
| CDC-2593  | B          | KY    | 1979 | AB     | B1            | plasmid                |                |                        | 245              |
| CDC-2746  | B          | NY    | 1979 | IB     | B1            | plasmid                |                |                        | 245              |
| CDC-2978  | B          | CO    | 1979 | IB     | Bbv          | plasmid                |                |                        | 170              |
| CDC-4848⁵ | B          | ATCC  | 2576S| Bnp    |              | plasmid                |                |                        | 55               |
| CDC-5078  | B          | HI    | 1983 | IB     | Bbv          | plasmid                |                |                        | 245              |
| CDC-5153  | B          | IN    | 1984 | IB     | B1            | plasmid                |                |                        | 139              |
| CDC-5168  | B          | HI    | 1984 | IB     | Bbv          | plasmid                |                |                        | 245              |
| CDC-5250  | B          | LA    | 1984 | IB     | B1            | plasmid                |                |                        | 245              |
| CDC-5281  | B          | OK    | 1984 | IB     | B2            | chromosome             |                |                        |                  |
| CDC-5323  | B          | DE    | 1985 | IB     | B1            | plasmid                |                |                        | 245              |
| CDC-7699  | B          | LA    | 1990 | FB     | B1            | plasmid                |                |                        | 245              |
| CDC-7827  | B          | NV    | 1991 | IB     | B1            | n.d. ⁶                 |                |                        | 245              |
| MDb02     | B          | -     | -    | -      | B1            | plasmid                |                |                        | 245              |
| ISS-BC1   | B          | I     | 2000 | olives | B2            | chromosome             |                |                        |                  |
| ISS-BC2   | B          | I     | 2000 | olives | B2            | chromosome             |                |                        |                  |
| ISS-BC3   | B          | I     | 2001 | truffles | B2         | chromosome             |                |                        |                  |
| ISS-251   | B          | I     | 2003 | chickpea | B2       | chromosome             |                |                        |                  |
| ISS-257   | B          | I     | 2002 | FB     | B2            | chromosome             |                |                        |                  |
| ISS-267   | B          | I     | 2003 | IB     | B2            | chromosome             |                |                        |                  |
| ISS-274   | B          | I     | 2004 | FB     | B2            | chromosome             |                |                        |                  |
| ISS-276   | B          | I     | 2002 | honey  | B2            | chromosome             |                |                        |                  |
| ISS-306   | B          | I     | 2004 | FB     | B2            | chromosome             |                |                        |                  |
| ISS-310   | B          | I     | 2004 | FB     | B2            | n.d. ⁶                 |                |                        |                  |
| ISS-331   | B          | I     | 2004 | IB     | B2            | chromosome             |                |                        |                  |
| ISS-333   | B          | I     | 2004 | FB     | B2            | plasmid                |                |                        | 245              |
bivalent *C. botulinum* type Ab strain (ISS-87); and 1/21 (5%) *bont* /B subtypes.

When the Southern blots of the PFGE gels containing the genomic DNA from the bivalent *C. botulinum* strains included in this study were stripped and rehybridized with a *bont*/A specific gene probe, a hybridization signal was observed at the same blot location that reacted with the *bont*/B specific gene probe, indicating that the *bont*/A and /B genes share the same genomic location in these strains. Specifically, both *bont*/A and /B probes hybridized to the chromosomal band of 6 bivalent *C. botulinum* strains, including all 5 *C. botulinum* type A(B) strains and 1 of the 4 *C. botulinum* type Ab strains (CDC-588): all 6 strains exhibited the bivalent *bont*/B PCR-RFLP subtype and had previously been shown to possess the *bont*/A1 subtype [17,20]. In the remaining 3 bivalent *C. botulinum* type Ab strains (CDC-1436, ISS-87, and ISS-92), both *bont*/A and /B probes hybridized to the same extra-chromosomal blot location: the 3 strains exhibited *bont*/B1, /B3, and bivalent/B PCR-RFLP subtypes, respectively, and had previously been shown to possess the *bont*/A2 subtype [17,20] (Table 1).

Figure 2 shows a PFGE gel stained with ethidium bromide and its Southern blot membrane after hybridization with a non-radioactive *bont*/B gene probe.

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**Table 1. cont.**

| Strain no | Toxin type | State | Year | Source | *bont*/B subtype | *bont*/B genomic location | *bont*/A subtype | *bont*/A genomic location | Plasmid size (kb) |
|-----------|------------|-------|------|--------|-----------------|-------------------------|-----------------|-------------------------|-----------------|
| ISS-338   | B          | I     | 2002 | honey  | B2             | chromosome             |                 |                         |                 |
| ISS-342   | B          | I     | 2004 | tuna fish | B2         | chromosome             |                 |                         |                 |
| ISS-360   | B          | I     | 2005 | peppers | B2           | chromosome             |                 |                         |                 |
| ISS-372   | B          | I     | 2005 | beans   | B2           | chromosome             |                 |                         |                 |
| ISS-378   | B          | I     | 2006 | mushrooms | B2       | chromosome             |                 |                         |                 |
| ISS-388   | B          | I     | 2006 | FB      | B2           | chromosome             |                 |                         |                 |
| CDC-588   | Ab         | OH    | 1976 | FB      | Bbv          | chromosome             | A1              | chromosome             | 245             |
| CDC-1436  | Ab         | UT    | 1977 | IB      | Bbv          | plasmid                 | A2              | plasmid                 | 245             |
| ISS-87    | Ab         | I     | 1995 | FB      | B3           | plasmid                 | A2              | plasmid                 | 245             |
| ISS-92    | Ab         | I     | 1993 | FB      | B1           | plasmid                 | A2              | plasmid                 | 245             |
| CDC-1634  | A(B)       | PA    | 1977 | IB      | Bbv          | chromosome             | A1              | chromosome             |                 |
| CDC-1727  | A(B)       | AK    | 1977 | whale oil | Bbv       | chromosome             | A1              | chromosome             |                 |
| CDC-1807  | A(B)       | CO    | 1977 | beans and franks | Bbv  | chromosome             | A1              | chromosome             |                 |
| CDC-4893  | A(B)       | IL    | 1983 | FB      | Bbv          | chromosome             | A1              | chromosome             |                 |
| CDC-5277  | A(B)       | WV    | 1984 | IB      | Bbv          | chromosome             | A1              | chromosome             |                 |

1CDC (Centers for Disease Control and Prevention, USA); ISS (Istituto Superiore di Sanità, Italy).
2IB (infant botulism); FB (foodborne botulism); AB (animal botulism); uB (unknown botulism).
3Bnp (non-proteolytic B); Bbv (bivalent B).
4The size of the closest molecular standard bands are indicated.
5Non-proteolytic *C. botulinum*.
6n.d. = Not determined, because of DNA degradation.
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**Figure 1.** BamHI, HindIII, Sau and EcoRV restrictions of the *bont*/B PCR products obtained from strains CDC-1758 (lanes 1, 5, 9, 14); CDC-1828 (lanes 2, 6, 10, 15); CDC-1436 (lanes 3, 7, 11, 16); CDC-4848 (lanes 4, 8, 12); CDC-816 (lane 13). M.S. (Molecular standard, 1 kb Promega).
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Characterization and size determination of the extra-chromosomal bands

The PFGE Southern blot assay can also be used to estimate the size of extra-chromosomal hybridization bands, when present; however, large circular DNA molecules can exhibit anomalous migration during PFGE [21,22]. As shown in Figure 3a, the DNA preparations from several C. botulinum isolates displayed double bands after hybridization with the bont/B gene probe: this could indicate either that these isolates carry multiple copies of the bont/B gene on two distinct extra-chromosomal elements of different sizes or that the same extra-chromosomal element harboring the bont/B gene exists as variable forms whose mobility differs under PFGE conditions. The latter hypothesis is consistent with the diverse mobility of the isoforms of large (>100 kb) circular plasmids: i) open circular forms, which remain trapped in the sample wells of pulsed-field gels; ii) closed supercoiled forms, which move slowly under PFGE conditions; and iii) linear forms, which migrate at rates that allow the size of the plasmids to be accurately determined [22]. Based on these considerations, the double extra-chromosomal bands that we observed in some DNA samples might correspond to the linear and supercoiled forms of the same plasmids. To test this hypothesis, the PFGE plugs were treated with S1 nuclease, which enzymatically converts all plasmid forms into linear forms [22]. The S1 nuclease treatment caused the disappearance of the slower of the two bands present in the DNA samples, indicating that this band was probably the supercoiled plasmid; the mobility of the faster band was not affected by S1 nuclease treatment, which is consistent with the behavior expected for the linear plasmid [22]. Whether or not linear and supercoiled plasmid forms are present depends on the age of the culture [22], which is consistent with the appearance of the double hybridization bands in some, but not all, of our DNA samples: in fact, the DNA preparations that we used were obtained from bacteria collected from overnight cultures, whose growth phases were not uniform (see the Materials and Methods section). Other DNA samples displayed single extra-chromosomal bands after hybridization with the bont/B gene, which presumably corresponded to the linear plasmid forms; indeed, their mobility did not change after S1 nuclease treatment.

As illustrated in Figure 3b, PFGE resolved at least five differently sized bont/B-carrying plasmids among the 32 C. botulinum isolates. They ranged from ~55 kb to ~245 kb, as determined by comparison with a molecular standard (DNA isolated from Salmonella enterica serotype Braenderup strain H9812 and restricted with XbaI) [23]. Specifically, a plasmid greater than the 245 kb band of the molecular size marker was detected in 23 C. botulinum strains (72% of the 32 strains harboring a plasmid-borne bont/B gene). Of these, 16 displayed the bont/B1 PCR-RFLP subtype, 5 displayed the bivalent bont/B PCR-RFLP subtype, and 2 displayed the bont/B2 and bont/B3 PCR-RFLP subtypes (Table 4). A plasmid in line with the 139 kb band of the molecular standard was observed in 4/32 (12.5%) C. botulinum strains, all of which exhibited the bont/B1 PCR-RFLP subtype. Furthermore, plasmids close in size to the 170 kb and 55 kb bands of the

Table 3. Plasmid-borne bont/B PCR-RFLP subtype genes among C. botulinum strains.

| bont/B PCR-RFLP subtype | No. of PFGE-typeable strains (n = 60) | Plasmid carriage (%) (n = 32) |
|--------------------------|-------------------------------------|-------------------------------|
| B1                       | 22                                  | 21 (95%)                      |
| B2                       | 21                                  | 1 (5%)                        |
| B3                       | 2                                   | 1 (50%)                       |
| bivalent B               | 13                                  | 7 (54%)                       |
| nonproteolytic B         | 2                                   | 2 (100%)                      |

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molecular standard were observed in 2 \textit{C. botulinum} strains each; both \( \sim 170 \text{ kb} \) plasmids carried the bivalent \( \text{bont}/B \) PCR-RFLP subtype, whereas both \( \sim 55 \text{ kb} \) plasmids carried the non-proteolytic \( \text{bont}/B \) PCR-RFLP subtype. Finally, a unique plasmid of approximately \( 217 \text{ kb} \) was detected in a single strain displaying the \( \text{bont}/B1 \) PCR-RFLP subtype. The plasmids harboring both \( \text{bont}/A \) and \( /B \) genes that were detected in 3 bivalent \textit{C. botulinum} strains were \( > 245 \text{ kb} \).

### Discussion

Subtype diversity within BoNT serotypes can significantly impact their receptor binding, target affinity and antibody recognition; the latter issue may be critical for the development of effective neutralizing antibodies [3]. However, little is known about the distribution of the BoNT subtypes and their encoding genes among \textit{C. botulinum} strains with different origins. We previously analyzed the \( \text{bont}/A \) gene of a set of \textit{C. botulinum} strains using a PCR-RFLP approach [17]; although this method can only detect a few single nucleotide polymorphisms that are recognized by specific restriction enzymes, it correctly identified the \( \text{bont}/A \) subtype genes, as subsequently shown by the complete nucleotide sequencing of some of those genes [24,25]. In the current study, we adopted a similar PCR-RFLP approach for subtyping the \( \text{bont}/B \) gene in a panel of \textit{C. botulinum} strains from different origins (clinical forms of botulism and foods), countries, and periods. Our results revealed that the \( \text{bont}/B2 \) PCR-RFLP subtype was more prevalent among \textit{C. botulinum} strains from Italy, whereas the \( \text{bont}/B1 \) PCR-RFLP subtype prevailed among strains from the US, confirming earlier suggestions by Hill et al. [4]. A similar geographic distribution has been found for \( \text{bont}/A \) subtypes, with \( \text{bont}/A2 \) more prevalent in Italy and \( \text{bont}/A1 \) more prevalent in the US [17]. These results could reflect a variation in the evolutionary history of both \( \text{bont}/A \) and \( /B \) subtypes. Furthermore, no correlation was observed between any specific \( \text{bont}/B \) subtype and the clinical or food source of the strains or the period in which the strains were isolated, as previously shown for \( \text{bont}/A \) subtypes [17].

Unexpectedly, we found that the bivalent \( \text{bont}/B \) PCR-RFLP subtype was not restricted to the bivalent \textit{C. botulinum} strains and

### Table 4. Molecular size of the plasmids carrying the different \( \text{bont}/B \) subtype genes.

| Plasmid Size (kb)* | \( \text{bont}/B1 \) | \( \text{bont}/B2 \) | \( \text{bont}/B3 \) | bivalent \( \text{bont}/B \) | Nonproteolytic \( \text{bont}/B \) |
|-------------------|-------------------|-----------------|-----------------|-----------------|-----------------|
| \( \sim 245 \)    | 16                | 1               | 1               | 5               | –               |
| \( \sim 217 \)    | 1                 | –               | –               | –               | –               |
| \( \sim 170 \)    | –                 | –               | –               | 2               | –               |
| \( \sim 139 \)    | 4                 | –               | –               | –               | –               |
| \( \sim 55 \)     | –                 | –               | –               | 2               | –               |

*The plasmid sizes were deduced by comparison with a molecular standard [23].

Slight differences between the sizes of plasmids included in the same group were observed.

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was detected in several non-bivalent type B strains; we also found that two bivalent C. botulinum type Ab strains exhibited the bont/B1 and /B3 PCR-RFLP subtypes. These results could indicate that the bont/B genes of these peculiar C. botulinum strains could be previously unrecognized subtype genes, although this would need to be confirmed with sequencing. Alternatively, the finding that some C. botulinum strains display bont/B subtypes other than the expected ones could be indicative of mobilization of the bont/B gene among strains at some point during evolution. Circumstantial evidence that the bont/B gene is mobile and might have been transferred among progenitor strains already exists, in particular: i) BoNT/B can be formed by C. botulinum strains of different clostridia groups [2]; ii) BoNT/B is produced along with another BoNT type by some C. botulinum strains, such as those of type Ab, Ba, and Bf [16]; iii) a silent bont/B gene is present in the genome of C. botulinum type A(B) strains [15]; and iv) toxigenic C. botulinum type B and its non-toxigenic derivatives have been isolated from the same samples [26]. Although there is no direct evidence of the mobilization of the bont/B gene, the recent demonstrations that the gene is plasmid-encoded in certain C. botulinum strains further support this hypothesis: indeed, plasmids may play an important role in mediating genetic transfer within and among bacterial genomes [27].

With regard to the genomic location of the bont/B1, /B3 and bivalent /B subtype genes atypically detected in the C. botulinum strains mentioned above, our results showed that the bont/B genes were extra-chromosomally located in all of these strains, though for one strain the bivalent bont/B subtype gene could not be localized because of consistent DNA degradation. Analysis of the genomic location of the bont/B genes was then extended to all C. botulinum strains included in this study: surprisingly, extra-chromosomal elements carrying the bont/B gene were detected in most (53%), with no apparent relationship with their origin.

We assumed that the bont/B-carrying extra-chromosomal elements were circular plasmids, based on the agreement between their PFGE migration and the mobility behavior predicted for the supercoiled and linear forms of circular plasmids [22]. Furthermore, some of the extra-chromosomal elements were similar in size to those of the bont/B-encoding plasmids determined to date [i.e., 270 kb for pCLJ of strain 657, 149 kb for pCLD of strain Okra [12], and 48 kb for pCLL of strain Eklund 17B (GenBank Accession number CP001057)], though two previously unreported sizes were also revealed in this study. However, we predicted the plasmid sizes by comparison with a molecular standard, whereas an accurate size determination would require the complete nucleotide sequencing of the plasmids.

Our results indicate that all bont/B subtypes can be located on plasmids and that a C. botulinum strain can only carry bont/B plasmids of a single size. Specifically, of the bont/B plasmids identified for 32 C. botulinum strains, 24 were greater than 200 kb, and the remaining 8 ranged from approximately 55 kb to ~170 kb: assuming that the C. botulinum chromosome is about 3.9 Mb [9], such plasmids would constitute a variable proportion (from 0.1% to 6%) of the genomes of the strains harboring them. The largest plasmids (~245 kb) were associated with all bont/B subtypes, except for the non-protectolytic bont/B subtypes, which were only associated with the smallest plasmids (~55 kb). Notably, one of the non-protectolytic bont/B subtypes belonged to strain CDC-4884 (or ATCC 25765), which was found to correspond to strain Eklund 17B: this strain has plasmid sequence of ~48 kb (GenBank Accession number CP001057), which is similar to the ~55 kb in our study. The intermediate-sized plasmids detected in the present study were associated with either the bont/B1 or the bivalent bont/B subtypes, suggesting that both subtypes can reside on plasmids of different sizes. For the strains whose bont/B gene was plasmid-borne, no chromosomal band hybridized with the bont/B gene probe, indicating that the plasmids did not integrate with the chromosome.

Remarkably, 95% of the bont/B1 subtypes resided on plasmids, whereas the same percentage of the bont/B2 subtypes resided on the chromosome. Although the biological significance of this finding is unclear, it supports the hypothesis of diverse evolutionary pathways for the bont/B1 and /B2 subtypes, as hypothesized above based on their geographic separation.

For three of the nine bivalent C. botulinum strains (all three of type Ab), the bont/A and /B genes were located on the same plasmid, whose size was similar to that of plasmid pCLJ (270 kb) of the bivalent C. botulinum type Ba strain 657 [10,12]. However, for the other 6 bivalent C. botulinum strains [one of type Ab and 5 of type AB], the bont/A and /B genes were located in the chromosome. The finding that the bont/A and /B genes share the same genomic location (whether it was the chromosome or the plasmid) suggests that they are structurally linked. The specific location could be related to the bont/A subtype: in fact, all three of the strains showing plasmid location of the genes had bont/A2 and all 6 of the bivalent strains with chromosome location had bont/A1 [17,20]. However, this hypothesis would need to be tested with more bivalent strains.

To determine the reasons for which the genomic location of the bont/A and /B genes varies among the different C. botulinum strains, the bont plasmids identified in the present study will need to be characterized at the sequence level. Partial mobile-enabling sequences, such as insertion sequences (IS), have been detected downstream of the bont/B and /A genes in plasmids pCLJ, pCLD and pCLK; when chromosomal, the bont genes are flanked by both upstream and downstream IS-like elements, which might be indicative of transposon integration [12]. The association between remnants of IS elements and the bont/A and /B genes suggests that these genes could have been mobilized and stably inserted into the chromosome or plasmid, depending on the recipient Clostridium ancestor strain [28]. The same mechanism might have contributed to the dissemination of the bont genes among heterogeneous groups of clostridia; in this respect, the ability of the BoNT-encoding plasmids to undergo conjugation or other types of genetic transfer should also be investigated.

Notably, many similarities have previously been reported among the BoNT/B of C. botulinum, the BoNT/G of C. argentinense, and the tetanus neurotoxin (TeNT) of C. tetani, which is also plasmid-encoded [29]: in particular, these neurotoxins have a high nucleotide and amino-acid sequence homology [4,30], and they cleave the same presynaptic membrane protein synaptobrevin, though at different peptide bonds [1]. Furthermore, BoNT/B and /G recognize the same neuronal receptors [31,32]. The finding that the bont/B gene can be plasmid-borne, like the bont/G and tent genes, strongly supports the hypothesis that they descend from a common ancestor.

A future challenge will be to determine whether the BoNT-encoding plasmids play a role in the development and/or flexibility of C. botulinum, thus potentially increasing its adaptability to certain niches, such as specific environments and food matrices, and ultimately contributing to the disease of botulism.

**Materials and Methods**

**Clostridia strains and culture conditions**

A total of 63 C. botulinum strains were used in this study (Table 1). Of these, 20 were from the culture collection of the National Reference Center for Botulism, Istituto Superiore di Sanita’ (ISS),
BoNT/B and were positive for the treatment of plugs

Pulsed-field gel electrophoresis (PFGE) and S1 nuclease PCR restriction fragment length polymorphism (RFLP)

1% L-cysteine hydrochloride monohydrate) and grown overnight at (5% Trypticase, 0.5% peptone, 0.4% glucose, 2% yeast extract, yolk agar (EYA) plates (Oxoid, Basingstoke, England). Single cultures were prepared as previously described [17]. The DNA from distinct cases of human botulism and 1 from animal botulism; 1 strain (ATCC 25765) was from a marine sediment (Aspergillus oryzae) with 1 unit of *A. oryzae* S1 nuclease (MBI Fermentas, Vilnius, Lithuania) in 200 μl of S1 buffer for 10 min at 37°C. PFGE of undigested or S1-digested DNA was carried out in a contour-clamped homogeneous electric field apparatus (CHEF Mapper apparatus, BioRad Laboratories, Hercules, CA). A constant temperature of 14°C was used, and the electrophoresis parameters were as follows: voltage of 6 V/cm, an angle of 120, and switch times of 4 to 40 sec (linear ramping factor), for 22 hr. Gels were stained with ethidium bromide and visualized in a GelDoc 2000 apparatus (Bio-Rad Laboratories).

Probes preparation and Southern hybridization

A 592 bp fragment of the *bont/B* gene and a 268 bp fragment of the *bont/A* gene were PCR labeled with digoxigenin (DIG), using primers described elsewhere [10] and a non-radioactive DNA probe labeling kit (PCR DIG Probe Synthesis Kit, Roche Diagnostics GmbH, Mannheim, Germany). The DNA was transferred from PFGE gels to positively charged nylon membranes (Hybond-N+, Roche) by overnight capillary transfer with buffer 20× SSC (3.0 M sodium chloride, 0.3 M sodium citrate, pH 7.0). After transfer, nylon membranes were hybridized with either *bont/B* or *bont/A* gene probes at 42°C for 18 h in a DIG Hyb Solution (Roche).

A chemiluminescence-based method was used to detect probe-target hybrids, according to the manufacturer’s instructions (Roche). Briefly, the membranes were blocked for 30 min; 20 μl of anti-digoxigenin-AP Fab-frags (15 μg/μl) (Roche) were added to the blocking solution; and the membranes were incubated for 30 min at room temperature. After equilibration in detection buffer, the membranes were incubated with chemiluminescent substrate CSPD in a Hypercassette (Amersham Pharmacia Biotech, Milan, Italy) and exposed to CL-XPosure film (Pierce Chemical, Rockford, IL).

For membrane stripping and rehybridization, a previously hybridized membrane was rinsed with distilled water and then soaked three times for 30 min in 0.2 M NaOH containing 0.1% sodium dodecyl sulfate at 37°C, to remove the bound probe. The membrane was washed for 15 min in 2× SSC and then hybridized with a second probe.

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Author Contributions

Concepted and designed the experiments: GF. Performed the experiments: AM CS. Analyzed the data: GF AM CS. Wrote the paper: GF PA.

References

1. Rossetto O, Seveso M, Caccin P, Schiavo G, Montecucco C (2001) Tetanus and botulinum neurotoxins turning bad guys into good by research. Toxicon 39: 27–41.
2. Lindstrom M, Korkeala H (2006) Laboratory diagnostics of botulism. Clin Microbiol Rev 19: 290–314.
3. Smith TJ, Lou J, Geren IN, Forsyth CM, Tsai R, et al. (2005) Sequence variation within botulinum neurotoxin serotypes impacts antibody binding and neutralization. Infect Immun 73: 5450–5457.
4. Hill KK, Smith TJ, Helma CH, Ticknor LO, Foley BT, et al. (2007) Genetic diversity among botulinum-neurotoxin producing clostridial strains. J Bacteriol 189: 4810–4821.
5. Inoue K, Iida H (1970) Conversion of toxigenicity in *Clostridium botulinum* type C. Jpn J Microbiol 14: 87–89.
6. Eklund MW, Poskpyt FT, Reed SM (1972) Bacteriophage and the toxigenicity of *Clostridium botulinum* type D. Nat New Biol 233: 16–17.
7. Eklund MW, Pesky FT, Mestril LM, Strom MS (1988) Evidence for plasmid-mediated toxin and bacteriocin production in Clostridium botulinum type G. Appl Environ Microbiol 54: 1405–1408.
8. Zhou Y, Sugiyama H, Nakano H, Johnson EA (1995) The genes for the Clostridium botulinum type G toxin complex are on a plasmid. Infect Immun 63: 2087–2091.
9. Sebaihia M, Peck MW, Minton NP, Thomson NR, Holden MY, et al. (2007) Genome sequence of a prototypic Group I Clostridium botulinum strain Hall A and comparative analysis of the clostridial genomes. Genome Res 17: 1082–1092.
10. Marshall KM, Bradshaw M, Pellett S, Johnson EA (2007) Plasmid encoded neurotoxin genes in Clostridium botulinum serotype A subtypes. Biochem Biophys Res Commun 361: 49–54.
11. Hatheway CL, McCroskey LM, Lombard GL, Dowell VR Jr (1981) Atypical toxin variant of Clostridium botulinum type B associated with infant botulism. J Clin Microbiol 14: 607–611.
12. Smith TJ, Hill KK, Foley BT, Detter JC, Munk AC, et al. (2007) Analysis of the neurotoxin complex genes in Clostridium botulinum A1–A4 and B1 strains: BoNT/A3, /Ba4 and /B1 clusters are located within plasmids. PloS ONE 2: e1271. doi:10.1371/journal.pone.0001271.
13. Terre H (1999) Botulism in the European Union. Euro Surveill 4: 2–7.
14. Centers for Disease Control and Prevention (1998) Botulism in the United States, 1899–1996. Handbook for epidemiologists, clinicians, and laboratory workers. Atlanta, GA: Centers for Disease Control and Prevention.
15. Franciosa G, Ferreira JL, Hatheway CL (1994) Detection of type A, B and E botulism neurotoxin genes in Clostridium botulinum and other Clostridium species by PCR—evidence of unexpressed type B toxin genes in type A toxigenic organisms. J Clin Microbiol 32: 1911–1917.
16. Giménez DF, Giménez JA (1995) The typing of botulinal neurotoxins. Int J Food Microbiol 27: 1–9.
17. Franciosa G, Floridi F, Maugliani A, Aureli P (2004) Differentiation of the gene clusters encoding botulinum neurotoxin type A complexes in Clostridium botulinum type A, Ab, and A(B) strains. Appl Environ Microbiol 70: 7192–7199.
18. Wang X, Maegawa T, Karasawa T, Kozaki S, Tsukamoto K, et al. (2000) Genetic analysis of type E botulinum toxin-producing Clostridium butyricum strains. Appl Environ Microbiol 66: 4992–4997.
19. Li J, Mizamoto K, McClane BA (2007) Comparison of virulence plasmids among Clostridium perfringens type E isolates. Infect Immun 75: 1811–1819.
20. Kirma N, Ferreira JL, Baumstark BR (2004) Characterization of six type A strains of Clostridium botulinum that contain type B toxin gene sequences. FEMS Microbiol Lett 16: 159–164.
21. Smírke JS, Scherer S (1999) Pulsed-field gel electrophoresis of circular DNA. Nucleic Acids Res 17: 4359–4365.
22. Barton BM, Harding GP, Zuccarelli AJ (1993) A general method for detecting and sizing large plasmids. Anal Biochem 226: 235–240.
23. Hunter SB, Vauterin P, Lambert-Fair MA, Van Duyne MS, Kobota K, et al. (2005) Establishment of a universal size standard strain for use with the PulseNet standardized pulsed-field gel electrophoresis protocol: converting the national databases to new size standard. J Clin Microbiol 43: 1045–1050.
24. Franciosa G, Maugliani A, Floridi F, Aureli P (2006) A novel type A2 neurotoxin gene cluster in Clostridium botulinum strain Mascarpone. FEMS Microbiol Lett 261: 81–94.
25. Raphael BH, Lazquez C, McCroskey LM, Joseph LA, Jacobson MJ, et al. (2008) Genetic homogeneity of Clostridium botulinum type A1 strains with unique toxin gene clusters. Appl Environ Microbiol 74: 4390–4397.
26. Yamakawa K, Karasawa T, Kakimura H, Marnyuma H, Takahashi H, et al. (1997) Emergence of Clostridium botulinum type B-like nontoxogenic organisms in a patient with type B infant botulism. J Clin Microbiol 35: 2163–2164.
27. Kelly BG, Vespermann A, Bolton DJ (2008) The role of horizontal gene transfer in the evolution of selected foodborne bacterial pathogens. Food Chem Toxicol: doi:10.1016/j.fct.2008.02.006.
28. Bruggemann H (2005) Genomics of clostridial pathogens: implication of extrachromosomal elements in pathogenicity. Curr Opin Microbiol 8: 601–605.
29. Finn CW, Silver RP, Habig WH, Hardgree MC, Zou G, et al. (1984) The structural gene for tetanus neurotoxin is on a plasmid. Science 224: 881–884.
30. Campbell K, Collins MD, East AK (1993) Nucleotide sequence of the gene coding for Clostridium botulinum (Clostridium argentinense) type G neurotoxin: genealogical comparison with other clostridial neurotoxins. Biochim Biophys Acta 1216: 487–491.
31. Rummel A, Karnath T, Henke T, Bigalke H, Binz T (2004) Synaptotagmins I and II act as nerve cell receptors for botulinum neurotoxin G. J Biol Chem 279: 30865–30870.
32. Rummel A, Eichner T, Weil T, Karnath T, Gutraiz A, et al. (2007) Identification of the protein receptor binding site of botulinum neurotoxin B and G proves the double-receptor concept. Proc Natl Acad Sci 104: 359–364.