AEM
Journals.ASM.org

Plasmid-Borne Type E Neurotoxin Gene Clusters in *Clostridium botulinum* Strains

Zhen Zhang, Hannamari Hintsa, Ying Chen, Hannu Korkeala, Miia Lindström

Department of Food Hygiene and Environmental Health, Centre of Excellence in Microbial Food Safety Research, Faculty of Veterinary Medicine, University of Helsinki, Helsinki, Finland

A collection of 36 *Clostridium botulinum* type E strains was examined by pulsed-field gel electrophoresis (PFGE) and Southern hybridization with probes targeted to *botE* and *orfX1* in the neurotoxin gene cluster. Three strains were found to contain neurotoxin subtype E1 gene clusters in large plasmids of about 146 kb in size.

Botulinum neurotoxin (BoNT) is the most potent natural toxin, produced by *Clostridium botulinum* and some strains of *Clostridium butyricum* and *Clostridium baratii*. The toxin blocks neurotransmitter release in cholinergic nerve ends, causing flaccid paralysis known as botulism (1). Based on their antigenic properties, BoNTs are classified into types A to G, of which toxins A, B, E, and F cause botulism in humans. Moreover, sequence analysis and binding studies with monoclonal antibodies have revealed several subtypes within most neurotoxin serotypes (2–6).

The genomic location of the neurotoxin gene cluster is variable and partly serotype dependent. In the early 1970s, the *botC* and *botD* clusters were observed to be encoded by bacteriophages (7). Recent genomic studies revealed that their prophages exist as large plasmids (8, 9). The *botG* cluster is carried by a plasmid (10). The *botA*, *botB*, and *botF* clusters were initially found in the bacterial chromosome; however, plasmid-borne toxin genes have recently been shown for subtypes A3 (11), B1, B2, the nonproteolytic type B (12–14), and some bivalent subtypes, such as Ab, Ba, and Bf (15). The two available *C. botulinum* type E genome sequences (NCBI) show chromosomal *botE* clusters, as is the case with neurotoxigenic *C. butyricum* (16). Type E neurotoxin genes carried by extrachromosomal elements have not been reported.

Here we evaluated the genomic location of the *botE* gene cluster in 36 *C. botulinum* type E strains isolated from aquatic environments, fish, and fish products (Table 1). All strains were incubated anaerobically in 10 ml of tryptone-peptone-glucose-yeast extract (TPGY) medium at 30°C. Agarose plugs containing genomic DNA of *C. botulinum* type E strains were prepared as described previously (1) and analyzed by pulsed-field gel electrophoresis (PFGE) in a 1% agarose gel. All samples including chromosomal DNA showed migration to a short distance. Smeared backgrounds were observed for some strains in the PFGE gel, indicating that the genomic DNA had degraded to some degree. This is probably due to the high levels of endogenous DNases produced by *C. botulinum* type E strains (1). In the gel lanes, DNA bands ranging in size from 9 to 146 kb were shown for 11 strains, including 250, CB11/1-1, K51, K22, K15, K101, K119, K23, K25, K28, and K8 (Fig. 1A), suggesting the presence of extrachromosomal elements. To measure the size of the potential plasmids accurately, the DNA embedded in PFGE plugs were incubated with S1 nuclease (Promega, Madison, WI) at 37°C for 45 min prior to electrophoresis to linearize the supercoiled plasmid DNA (17). However, for an unknown reason, S1 nuclease caused degradation of the genomic DNA of *C. botulinum* type E. Therefore, the sizes of the extrachromosomal elements were estimated based on non-S1-treated DNA and may thus represent supercoiled plasmids, making the size estimate inaccurate.

To further define the extrachromosomal elements, DNA of the PFGE gels was transferred to charged nylon membranes (Roche Applied Science, Mannheim, Germany) and hybridized with digoxigenin (DIG)-labeled 16S *rrn* probe. DIG-labeled hybridization probes were prepared by PCR labeling using primers listed in Table 2. Strong signals were observed for the large genomic DNA bands, whereas no hybridization signal with 16S *rrn* probe was observed for the smaller bands (Fig. 1B), suggesting that these elements represented plasmids. These results suggest that 30% of the *C. botulinum* type E strains studied harbor one or more plasmids ranging in size from 9 to 146 kb. The proportion is consistent with our previous study of 54 *C. botulinum* type E isolates (18). Of the 11 plasmid-containing strains, 8 represented the *botE* subtype E1 (Table 1).

To test whether any of the detected plasmids included the neurotoxin genes, the transfer membranes were stripped and subsequently rehybridized with probes specific for *botE* and finally with those specific for *orfX1*. Specific and distinct hybridization signals with the *botE* probe, consistent with the hybridization signals obtained with the 16S *rrn* probe, were observed for the chromosomal DNA from all but three strains, suggesting *botE* is in a chromosomal locus in these strains (Fig. 1C). In contrast, for strains CB11/1-1, K51, and K22, the *botE* probe hybridization signals were observed in extrachromosomal bands of approximately 146 kb in size, indicating their *botE* gene is located in a large plasmid (Fig. 1C). This finding was further confirmed by stripping and rehybridization with the *orfX1* probe (Fig. 1D). The strains with plasmid-borne *botE* were analyzed at least in triplicate (see Fig. S1 in the supplemental material). These results suggest that *C. botu-*

Received 11 January 2013 **Accepted** 28 March 2013
Published ahead of print 5 April 2013
Address correspondence to Miia Lindström, miia.lindstrom@helsinki.fi.
Supplemental material for this article may be found at http://dx.doi.org/10.1128/AEM.00080-13.

doi:10.1128/AEM.00080-13

TABLE 1 *Clostridium* strains used in this study

Strain	Lane no. in PFGE gel	Origin	Country	Yr	Source[a]	*botE* subtype
250	1	Canned salmon	UK	1978	Crowther/Lindroth[b]	ND[c]
CB11/1-1	2	Whitefish roe	Finland	1999	DFHEH	E1
K51	3	Rainbow trout surface	Finland	1996	DFHEH	E1
Beluga	4	Fermented white whale flippers	USA	1951	Dolman/Lindroth[b]	E1
K22	5	Burbot surface	Finland	1996	DFHEH	E1
341	6	European river lamprey	Finland	2005	DFHEH	E6
350	7	European river lamprey	Finland	2005	DFHEH	ND
4062	8	Muktuk	USA	1981	Hatheway/Lindroth[b]	ND
92	9	Marine environment	USA	1960s	Eklund/Lindroth[b]	ND
BL81/31	10	Smoked salmon	Canada	1934	IFR	E1
BL86/21	11	Fish	ND	ND	IFR	ND
BL87/1	12	Fish	USA	ND	IFR	ND
BL93/7	13	Fish	USA	ND	IFR	E1
BL93/8	14	Human	USA	ND	IFR	E2
C60	15	Dried mutton	Denmark	1989	SSI	ND
K15	16	Rainbow trout intestines	Finland	1995	DFHEH	E1
K101	17	Chub surface	Germany	1997	DFHEH	E1
K117	18	Smoked Canadian whitefish	Germany	1997	DFHEH	E3
K119	9	Rainbow trout intestines	Finland	1996	DFHEH	E1
K126	20	Smoked Alaskan salmon	Finland	1996	DFHEH	E3
K23	21	Burbot intestines	Finland	1996	DFHEH	ND
K25	22	Burbot surface	Finland	1996	DFHEH	E1
K28	23	Burbot surface	Finland	1996	DFHEH	ND
K3	24	Rainbow trout surface	Finland	1995	DFHEH	E3
K35	25	Vendace	Finland	1996	DFHEH	E6
K36	26	Smoked rainbow trout	Finland	1996	DFHEH	E6
K37	27	Smoked whitefish	Finland	1996	DFHEH	E1
K44	28	Rainbow trout intestines	Finland	1996	DFHEH	E3
K47	29	Rainbow trout surface	Finland	1996	DFHEH	ND
K7	30	Rainbow trout intestines	Finland	1995	DFHEH	E6
K76	31	Hot-smoked vendace	Finland	1997	DFHEH	E1
K8	32	Rainbow trout intestines	Finland	1995	DFHEH	E1
KA2	33	Seola Creek strain	USA	ND	Riemann/Lindroth[b]	ND
M3	34	Vegetarian sausage	Finland	2001	DFHEH	E3
RS1	35	Pacific red snapper	USA	1983	Lindroth[b]	ND
S16	36	Fish pond sediment	Finland	1997	DFHEH	E6

[a] DFHEH, Department of Food Hygiene and Environmental Health, University of Helsinki, Helsinki, Finland; IFR, Institute of Food Research, Norwich, United Kingdom; SSI, Statens Serum Institut, Copenhagen, Denmark.
[b] Collected from John Crowther, Claude Dolman, Charles Hatheway, Melvin Eklund, and Hans Riemann by Seppo Lindroth (University of California, Davis).
[c] ND, no data available.

linum type E strains CB11/1-1, K51, and K22 carry their neurotoxin gene clusters in large plasmids.

All three strains carrying a plasmid-borne *botE* gene were isolated in the 1990s from fish in Finland (Table 1). CB11/1-1 was involved in a food botulism outbreak in Finland in 1999 (19) and was epidemiologically unrelated to K51 and K22. Previous genotyping studies showed CB11/1-1 and K51 cluster together and are genetically distant from K22 (20–22), suggesting that the *botE*-containing plasmids are carried by *C. botulinum* strains representing at least two distinct genetic backgrounds. Our in-house amplified fragment length polymorphism (AFLP) typing collection shows that CB11/1-1 is identical to the nontoxigenic *C. botulinum* strain H61 (96% similarity) in AFLP analysis with an 89% cutoff value (data not shown), suggesting lateral transfer of the *botE* plasmid between toxigenic and nontoxigenic strains. Strain K51 showed a close genetic relatedness (higher than 85% similarity) to the chromosomal *botE*-carrying strains K23 and K28 (22). It is not known if the plasmids detected in these two strains, being smaller than the *botE* plasmid of K51, show similarity to the *botE* plasmid or if these two strains obtained their chromosomal neurotoxin genes as a result of plasmid transfer and further integration into their chromosome. Further comparative genome study will provide more evidence to understand the role of *botE*-containing plasmids in the evolution of *C. botulinum*.

Previous studies suggested that the plasmids carrying neurotoxin subtype A3 or A4 genes were approximately 270 kb in size, while those carrying neurotoxin type B genes varied considerably in size, ranging from 55 to 245 kb (11, 12). However, all three *botE*-containing plasmids have a similar size of approximately 146 kb. Although a previous study on 13 *C. butyricum* isolates showed all strains to carry their *botE* in the chromosome, the PFGE image indicated that four isolates, including those from two infant botulism cases, might harbor plasmids ranging in size from 120 to 170 kb (16). Whether such large plasmids in *C. butyricum* show regions of homology to the *botE*-containing plasmids detected here requires further inves-

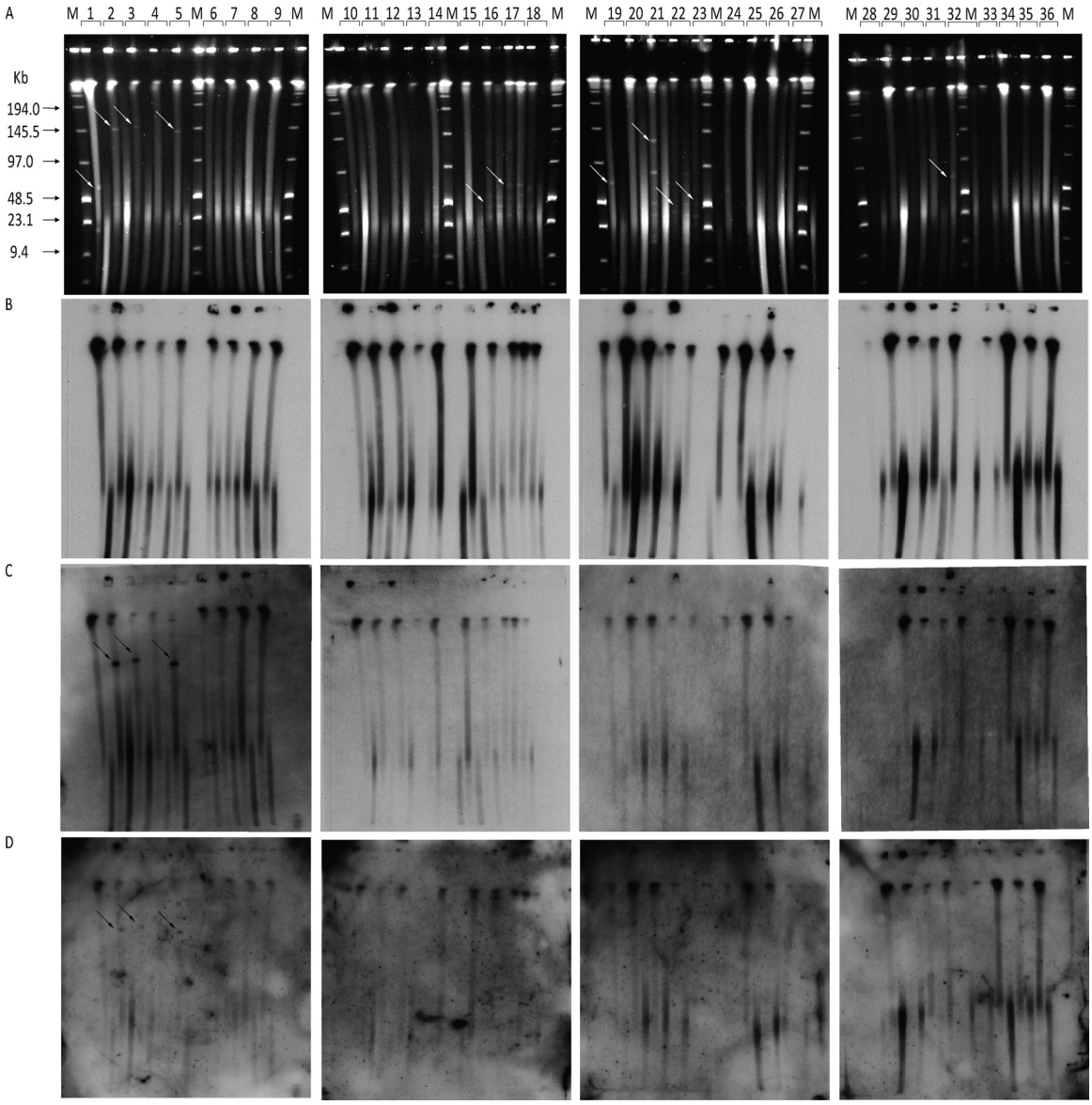

FIG 1 PFGE of nondigested genomic DNA from *C. botulinum* type E (A) and Southern hybridization with probes specific for 16S *rrn* (B), *botE* (C), and *orfX1* (D). Strain numbers correspond to those listed in Table 1. Each strain was loaded with genomic DNA (left) and S1 nuclease-treated genomic DNA (right) in two adjacent gel wells. For the strains shown in lanes 14, 23, 27, and 32, the marker (M) was loaded in the lane between the genomic DNA and S1 nuclease-treated genomic DNA. The white arrows indicate plasmid bands (A), and the black arrows indicate plasmid bands hybridized with probes specific for *botE* (C) and *orfX1* (D).

tigation. Mobility of plasmids is often mediated by conjugation mechanism (23). A recent study revealed that *bot*-containing plasmids were able to conjugatively transfer from strains CDC-A3, 657 Ba4, and Eklund 17Bnp to a nontoxigenic mutant of strain 62A1 (24). Further studies are warranted to evaluate if the *botE*-containing plasmids can be conjugatively transferred to other *C. botulinum* or *C. butyricum* strains.

In conclusion, we provide the first evidence of plasmid-borne type E neurotoxin gene clusters. All three plasmid-borne neurotoxin genes represented subtype E1 and were located within large plasmids of approximately 146 kb in size. These results may have important implications for understanding the evolution and toxigenicity of *C. botulinum* type E.

TABLE 2 Oligonucleotide primers used in this study

Primer	Sequence (5′→3′)
botE-F	TTTTTGTGGCTTCCGAGAAT
botE-R	TATTTTCACCTTCGGGCACT
orfX1-F	AAAATTACATTTATTGATGGTTACCTG
orfX1-R	AGAATTCCATTTTTAGTTATCCTTTTT
16S *rrn*-F	GAAGGCGACTTTCTGGACTG
16S *rrn*-R	TAAGGTTCTTCGCGTTGCTT

ACKNOWLEDGMENTS

This work was performed at the Centre of Excellence in Microbial Food Safety Research and was supported by the Academy of Finland (141140), Research Funds of the University of Helsinki, the European Community's Seventh Framework Programme CLOSTNET (237942), and the Finnish Veterinary Foundation.

We thank Hanna Korpunen for technical assistance.

REFERENCES

1. **Hielm S, Björkroth J, Hyytiä E, Korkeala H.** 1998. Genomic analysis of *Clostridium botulinum* group II by pulsed-field gel electrophoresis. Appl. Environ. Microbiol. **64**:703–708.
2. **Carter AT, Paul CJ, Mason DR, Twine SM, Alston MJ, Logan SM, Austin JW, Peck MW.** 2009. Independent evolution of neurotoxin and flagellar genetic loci in proteolytic *Clostridium botulinum*. BMC Genomics **10**:115. doi:10.1186/1471-2164-10-115.
3. **Chen Y, Korkeala H, Aarnikunnas J, Lindström M.** 2007. Sequencing the botulinum neurotoxin gene and related genes in *Clostridium botulinum* type E strains reveals *orfX3* and a novel type E neurotoxin subtype. J. Bacteriol. **189**:8643–8650.
4. **Dover N, Barash JR, Arnon SS.** 2009. Novel *Clostridium botulinum* toxin gene arrangement with subtype A5 and partial subtype B3 botulinum neurotoxin genes. J. Clin. Microbiol. **47**:2349–2350.
5. **Hill KK, Smith TJ, Helma CH, Ticknor LO, Foley BT, Svensson RT, Brown JL, Johnson EA, Smith LA, Okinaka RT, Jackson PJ, Marks JD.** 2007. Genetic diversity among botulinum neurotoxin-producing clostridial strains. J. Bacteriol. **189**:818–832.
6. **Smith TJ, Lou J, Geren IN, Forsyth CM, Tsai R, Laporte SL, Tepp WH, Bradshaw M, Johnson EA, Smith LA, Marks JD.** 2005. Sequence variation within botulinum neurotoxin serotypes impacts antibody binding and neutralization. Infect. Immun. **73**:5450–5457.
7. **Iida H, Oguma K, Inoue K.** 1974. Phage-conversion of toxigenicity in *Clostridium botulinum* types C and D. Jpn. J. Med. Sci. Biol. **27**:101–103.
8. **Sakaguchi Y, Hayashi T, Kurokawa K, Nakayama K, Oshima K, Fujinaga Y, Ohnishi M, Ohtsubo E, Hattori M, Oguma K.** 2005. The genome sequence of *Clostridium botulinum* type C neurotoxin-converting phage and the molecular mechanisms of unstable lysogeny. Proc. Natl. Acad. Sci. U. S. A. **102**:17472–17477.
9. **Skarin H, Håfström T, Westerberg J, Segerman B.** 2011. *Clostridium botulinum* group III: a group with dual identity shaped by plasmids, phages and mobile elements. BMC Genomics **12**:185. doi:10.1186/1471-2164-12-185.
10. **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.
11. **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.
12. **Franciosa G, Maugliani A, Scalfaro C, Aureli P.** 2009. Evidence that plasmid-borne botulinum neurotoxin type B genes are widespread among *Clostridium botulinum* serotype B strains. PLoS One **4**:e4829. doi:10.1371/journal.pone.0004829.
13. **Hill KK, Xie G, Foley BT, Smith TJ, Munk AC, Bruce D, Smith LA, Brettin TS, Detter JC.** 2009. Recombination and insertion events involving the botulinum neurotoxin complex genes in *Clostridium botulinum* types A, B, E and F and *Clostridium butyricum* type E strains. BMC Biol. **7**:66. doi:10.1186/1741-7007-7-66.
14. **Umeda K, Seto Y, Kohda T, Mukamoto M, Kozaki S.** 2009. Genetic characterization of *Clostridium botulinum* associated with type B infant botulism in Japan. J. Clin. Microbiol. **47**:2720–2728.
15. **Smith TJ, Hill KK, Foley BT, Detter JC, Munk AC, Bruce DC, Doggett NA, Smith LA, Marks JD, Xie G, Brettin TS.** 2007. Analysis of the neurotoxin complex genes in *Clostridium botulinum* A1-A4 and B1 strains: BoNT/A3, BoNT/Ba4 and BoNT/B1 clusters are located within plasmids. PLoS One **2**:e1271. doi:10.1371/journal.pone.0001271.
16. **Wang X, Maegawa T, Karasawa T, Kozaki S, Tsukamoto K, Gyobu Y, Yamakawa K, Oguma K, Sakaguchi Y, Nakamura S.** 2000. Genetic analysis of type E botulinum toxin-producing *Clostridium butyricum* strains. Appl. Environ. Microbiol. **66**:4992–4997.
17. **Barton BM, Harding GP, Zuccarelli AJ.** 1995. A general method for detecting and sizing large plasmids. Anal. Biochem. **226**:235–240.
18. **Hyytiä E, Hielm S, Björkroth J, Korkeala H.** 1999. Biodiversity of *Clostridium botulinum* type E strains isolated from fish and fishery products. Appl. Environ. Microbiol. **65**:2057–2064.
19. **Lindström M, Hielm S, Nevas M, Tuisku S, Korkeala H.** 2004. Proteolytic *Clostridium botulinum* type B in the gastric content of a patient with type E botulism due to whitefish eggs. Foodborne Pathog. Dis. **1**:53–57.
20. **Derman Y, Lindström M, Selby K, Korkeala H.** 2011. Growth of group II *Clostridium botulinum* strains at extreme temperatures. J. Food Prot. **74**:1797–1804.
21. **Keto-Timonen R, Nevas M, Korkeala H.** 2005. Efficient DNA fingerprinting of *Clostridium botulinum* types A, B, E, and F by amplified fragment length polymorphism analysis. Appl. Environ. Microbiol. **71**:1148–1154.
22. **Hielm S, Björkroth J, Hyytiä E, Korkeala H.** 1998. Prevalence of *Clostridium botulinum* in Finnish trout farms: pulsed-field gel electrophoresis typing reveals extensive genetic diversity among type E isolates. Appl. Environ. Microbiol. **64**:4161–4167.
23. **Smillie C, Garcillán-Barcia MP, Francia MV, Rocha EP, de la Cruz F.** 2010. Mobility of plasmids. Microbiol. Mol. Biol. Rev. **74**:434–452.
24. **Marshall KM, Bradshaw M, Johnson EA.** 2010. Conjugative botulinum neurotoxin-encoding plasmids in *Clostridium botulinum*. PLoS One **5**:e11087. doi:10.1371/journal.pone.0011087.