

# Diversity of Group I and II *Clostridium botulinum* Strains from France Including Recently Identified Subtypes

Christelle Mazuet<sup>1</sup>, Christine Legeay<sup>1</sup>, Jean Sautereau<sup>1</sup>, Laurence Ma<sup>2</sup>, Christiane Bouchier<sup>2</sup>, Philippe Bouvet<sup>1</sup>, and Michel R. Popoff<sup>1,\*</sup>

<sup>1</sup>Bactéries Anaérobies et Toxines, Institut Pasteur, Paris, France

<sup>2</sup>Plateforme Genomique-Pôle Biomics, Institut Pasteur, Paris, France

\*Corresponding author: E-mail: mpopoff@pasteur.fr.

Accepted: December 4, 2016

**Data deposition:** All the novel MLST alleles described in this study have been deposited in GenBank database: hsp60, KF681499 to KF681507; recA KF681489 to KF681498; rpoB, KF681478 to KF681488; oppB KF681460 to KF681477; acek, KF681440 to 681459; mdh, KF681425 to 681439; aroE, KF681410 to KF681424. GenBank accession numbers for the bont sequences type E12 (Mazuet, Sautereau, et al. 2015) were KF929215 and KM370319, for bontA7 (Becher et al. 2007) JQ954969, and for bontA8 KF667385.

## Abstract

In France, human botulism is mainly food-borne intoxication, whereas infant botulism is rare. A total of 99 group I and II *Clostridium botulinum* strains including 59 type A (12 historical isolates [1947–1961], 43 from France [1986–2013], 3 from other countries, and 1 collection strain), 31 type B (3 historical, 23 recent isolates, 4 from other countries, and 1 collection strain), and 9 type E (5 historical, 3 isolates, and 1 collection strain) were investigated by botulinum locus gene sequencing and multilocus sequence typing analysis. Historical *C. botulinum* A strains mainly belonged to subtype A1 and sequence type (ST) 1, whereas recent strains exhibited a wide genetic diversity: subtype A1 in *orfX* or *ha* locus, A1(B), A1(F), A2, A2b2, A5(B2') A5(B3'), as well as the recently identified A7 and A8 subtypes, and were distributed into 25 STs. *Clostridium botulinum* A1(B) was the most frequent subtype from food-borne botulism and food. Group I *C. botulinum* type B in France were mainly subtype B2 (14 out of 20 historical and recent strains) and were divided into 19 STs. Food-borne botulism resulting from ham consumption during the recent period was due to group II *C. botulinum* B4. Type E botulism is rare in France, 5 historical and 1 recent strains were subtype E3. A subtype E12 was recently identified from an unusual ham contamination. *Clostridium botulinum* strains from human botulism in France showed a wide genetic diversity and seems to result not from a single evolutionary lineage but from multiple and independent genetic rearrangements.

**Key words:** *Clostridium botulinum*, botulism, multilocus sequence typing, whole genome sequencing.

## Introduction

Botulinum neurotoxins (BoNTs) are the most potent toxins which are responsible for a rare but severe neurological disorder called botulism. The disease is characterized by a flaccid paralysis and decreased secretions which result from the BoNT-dependent inhibition of acetylcholine release at neuromuscular junctions and other cholinergic endings. BoNTs are divided into seven toxinotypes based on their antigenicity properties in neutralization assay (Popoff 1995; Peck et al. 2011; Barash and Arnon 2014; Dover et al. 2014). Thereby, neutralizing antibodies are specific of each toxinotype. All BoNT types induce similar pharmacological effects, which are characterized by flaccid paralysis, but with some differences between toxinotypes like duration and intensity of

symptoms. For example, BoNT/A induces the longest and most severe forms of botulism, compared with BoNT/E which leads to shorter duration symptoms and BoNT/B which is most often responsible for mild botulism illness (Eleopra et al. 1998; Keller et al. 1999; O'Sullivan et al. 1999; Foran et al. 2003; Meunier et al. 2003; Keller 2006). BoNT/B causes predominantly dysautonomic signs, whereas BoNT/A results in a pronounced paralytic effect on the respiratory muscles and most often leads to acute respiratory distress (Jenzer et al. 1975; Hughes et al. 1981; Merz et al. 2003; Sobel 2005; Potulska-Chromik et al. 2013). In addition, each toxinotype is subdivided into several subtypes according to amino acid sequence variations. Variability is observed in

*bont* genes, and amino acid sequence difference of at least 2.6% is assumed to define two distinct subtypes (Smith et al. 2005). Variations in BoNT amino acid sequences might affect some aspects of their activity such as binding to target cells, efficiency of entry into cells, potency of enzymatic activity, duration of effects, neutralization efficiency by antibodies specific of type and subtype. For example, BoNT/A2 enters cells more rapidly and more efficiently, and is a more potent neuromuscular blocker in vivo than BoNT/A1 (Pier et al. 2011; Torii et al. 2011). In addition, differences in in vitro and in vivo activity have been reported between subtypes BoNT/A1 to A5 using neuronal cell based and mouse assays, respectively (Whitemarsh et al. 2013). In contrast to BoNT/A1, A2, A4 and A5, the persistence of BoNT/A3 in rat spinal neurons is shorter (Whitemarsh et al. 2014). Moreover, antibodies against BoNT/A2 neutralize more efficiently BoNT/A2 than BoNT/A1, whereas antibodies against BoNT/A1 counteract efficiently both toxin subtypes (Torii et al. 2013). However, the impact of the subtypes in naturally acquired botulism is not yet well understood. Moreover, the recent controversy about the reported new BoNT type H, rather referred as a new hybrid F/A, is mainly based on the existence or absence of protection with antibodies against already known BoNT types and subtypes, and further raises the importance of subtype determination (Barash and Arnon 2014; Dover et al. 2014; Gonzalez-Escalona, Thirunavukkarasu, et al. 2014).

BoNTs are synthesized by *Clostridium botulinum* and atypical strains of *C. butyricum* and *C. baratii*. The *bont* genes are clustered with the genes of BoNT-associated nontoxic proteins (ANTPs) including hemagglutinins (HAs) or OrfX in a DNA fragment called the botulinum locus. The botulinum loci are located either on the chromosome, large plasmid, or phage depending on strains. *Clostridium botulinum* strains are heterogeneous at the phenotypic and genetic levels, and are classified into four groups (I–IV), group IV being assigned to a distinct species, *C. argentinense*. Two additional groups have been included, group V and VI which encompass BoNT/F-producing *C. baratii* strains, and BoNT/E-producing *C. butyricum* strains, respectively. In each group, *C. botulinum* strains exhibit genomic variability as evidenced by pulsed-field gel electrophoresis (PFGE), amplified fragment length polymorphism (AFLP), multilocus sequence typing (MLST), multilocus variable number tandem repeat analysis (MLVA), and single nucleotide polymorphism (SNP) based on whole genome sequencing (WGS) (review in Brüggemann et al. 2011; Hill and Smith 2013; Popoff and Bouvet 2013; Carter and Peck 2015; Smith, Hill, Raphael 2015). It is noteworthy that WGS is a powerful approach allowing not only to have access to detailed phylogenetic relatedness of neurotoxin-producing strains and toxin genes, but also to highlight the genomic modifications and genetic exchanges between strains (Peck et al. 2011; Smith, Hill, Xie, et al. 2015).

Human botulism is rare but recurrently identified in France. From 6 to 31 human cases of botulism (6–27 outbreaks) are

reported every year in our country (Carlier et al. 2007; Mazuet et al. 2011, 2014). Food-borne botulism is the most common form, and more especially botulism type B subsequently to the consumption of home-cured ham and pork products. However, since 2005, an increased number of botulism type A outbreaks was identified, which resulted in severe forms requiring hospitalization in intensive care units and mechanical respiratory ventilation for several weeks or months and one death was reported. Various food types were incriminated including home-made preparations, locally performed or imported from foreign countries, as well as industrial products. The incidence of food-borne botulism is variable in the European countries, and France with Germany, Spain and Italy are those with the highest number of reported cases within the period 1988–1998, not included the Eastern European countries (There 1999). Differences in food practices and notably in use of homemade canned foods likely account for the variable incidence of botulism in Europe. In contrast, infant botulism and wound botulism were more rare in France, 12 cases and 1 case, respectively, within the period 2004–2013 (Carlier et al. 2007; King et al. 2010; Mazuet et al. 2011, 2014). Infant botulism is prevalent in some countries such as United States and Argentina, whereas the incidence of this form of botulism is low in Europe. However, infant botulism is more frequent in Italy than in other European countries, 26 cases in the period 1984–2006 versus 1–9 cases in the other countries (Koepke et al. 2008). Differences in the dissemination of neurotoxicogenic *Clostridium* in the environment which seems to be the major source of contamination of the babies or differences in feeding practices, notably the distribution of honey to newborns in some countries (Aureli et al. 2002), likely reflect the differences in infant botulism incidence according to the countries. It is noteworthy that the comparison of epidemiological data from the different countries is not highly significant because the identification and notification of botulism cases are variable in each country.

The aim of this study is to analyze the genetic diversity of *C. botulinum* strains type A, B and E isolated in France in the recent years (1985–2013) versus historical strains (1947–1961) and some strains from other countries.

## Materials and Methods

### Bacterial Strains, Growth, Toxinotyping and DNA Preparation

The isolation of *C. botulinum* strains from clinical and food samples was performed with conventional culture method using fortified cooked meat medium (Becton Dickinson, France) and agar selective medium (Mazuet, Sautereau, et al. 2015). *Clostridium* strains were grown in Trypticase yeast extract glucose (TGY) broth in an anaerobic atmosphere at 37 °C. Phenotypic identification was performed with reference methods (Jousimies-Somer et al. 2002).

Toxin production and BoNT typing of each strain were determined by mouse test using anti-BoNT sera of the National Reference Center of Anaerobic bacteria and Botulism. All animal experiments were conducted with the approval of Institut Pasteur (agreement of laboratory animal use no 2013-0116). The presence of corresponding *bont* genes was confirmed using PCR methods as previously described (Vanhomwegen et al. 2013).

Total genomic DNA was extracted from *C. botulinum* cultures by lysozyme and proteinase K treatment as described previously (Dineen et al. 2003).

### PCR Amplification and DNA Sequencing

Overlapping pairs of primers covering the whole *bont* sequences were designed for PCR amplification using sequence data available in GenBank. PCR amplifications were performed in 50  $\mu$ l reaction buffer containing 200 ng DNA, 1  $\mu$ M of each primer (Sigma Aldrich, Germany), 1.5 mM MgCl<sub>2</sub>, 250 mM of each dNTP, 20 mM Tris-HCl, pH 8.4, 50 mM KCl and 1  $\mu$  Taq Polymerase (In Vitrogen, France). The PCR cycles consisted of 90 °C for 45 s, 50 °C for 45 s, and 72 °C for 1 min were repeated 30 times. A final extension step of 72 °C for 10 min was added. Amplicons were sequenced by EUROFINS/MWG, Germany.

### Whole Genome Sequencing

Whole genome sequencing using the NEBNext Ultra DNA Library Prep kit for Illumina (New England Biolabs) were performed using MiSeq machine (Illumina) in paired-end reads of 250 bases or on HiSeq2000 machine (Illumina) in single reads of 96 bases or 101 bases. Sequence files were generated using Illumina Analysis Pipeline version 1.8 (CASAVA). After quality filtering, reads were assembled using CLC software version 4 (CLC Bio).

### Multilocus Sequence Typing Analysis of Group I Strains

Multilocus sequence typing of group I strains was based on seven housekeeping genes (*aroE*, *mdh*, *aceK*, *appB*, *rpoB*, *recA*, and *hsp*) as previously described (Jacobson et al. 2008). Partial nucleotide sequences of the seven housekeeping genes were obtained by PCR amplification and subsequent sequencing according to the method described by Jacobson et al. (2008). For some strains, nucleotide sequences of housekeeping genes were deduced from WGS (table 1). MLST data were submitted to the *C. botulinum* MLST database (<http://pubmlst.org/cbotulinum/>, last accessed May 10, 2016). Allelic numbers and MLST sequence types (STs) were identified by querying *C. botulinum* MLST database. More than one nucleotide difference was regarded as a criterion for a different ST. New alleles and new STs were submitted to the *C. botulinum* MLST database.

### Multilocus Sequence Typing Analysis of Group II Strains

Multilocus sequence typing of group II strains was based on 12 housekeeping gene sequences (*oppB*, *pta*, *recA*, *rpoB*, *pyc*, *gyrB*, *lepA*, *ilvD*, *guaA*, *atpD*, *trpB*, *23S*) as described in (Macdonald et al. 2011; Weedmark et al. 2015). Allele sequences were concatenated and were aligned with Muscle (default parameters). Maximum Likelihood phylogenetic tree was built based on the Kimura 2-parameter model (Kimura 1980). About 1,000 bootstrap experiments were performed to assess the robustness of the topology and percent are indicated on nodes. Evolutionary analyses were conducted in MEGA (Tamura et al. 2013).

### Nucleotide Sequence Accession Numbers

All the novel MLST alleles described in this project have been deposited at GenBank database: *hsp60*, KF681499 to KF681507; *recA* KF681489 to KF681498; *rpoB*, KF681478 to KF681488; *oppB* KF681460 to KF681477; *aceK*, KF681440 to 681459; *mdh*, KF681425 to 681439; *aroE*, KF681410 to KF681424.

The project has been deposited at GenBank under the accession numbers KF929215 and KM370319 for *bont* sequences type E12 (Mazuet, Sautereau, et al. 2015), JQ954969 for *bont/A7* (Becher et al. 2007), and KF667385 for *bont/A8*.

## Results

### *Clostridium botulinum* Strains

The *C. botulinum* strains investigated in this study are listed in table 1. A total of 99 strains were analyzed including 59 *C. botulinum* type A (54 isolates from France, 4 from other countries, and 1 reference strain), 31 *C. botulinum* type B, and 9 *C. botulinum* type E strains (table 1).

The *C. botulinum* type A strains included 12 historical strains (Prévot's collection, 1947–1953) including 11 from French animal or food samples and 1 from Sweden, 1 reference strain isolated in 1976, as well as 46 strains isolated during the period 1986–2013: 26 strains were associated with food-borne botulism (13 outbreaks) in France and 1 in Switzerland, 14 with infant botulism (9 cases, France), 3 from food (France), 1 from a wound botulism in Switzerland, and 1 from cattle (Brazil) (table 1).

Three *C. botulinum* type B strains (historical strains) were from the Prévot's collection (1953–1961) including 2 from food-borne botulism and 1 from cattle botulism. A reference strain subtype B1 isolated in United Kingdom before 1947 is included. 23 *C. botulinum* type B strains were isolated in France during the period 1986–2013, 19 from food-borne botulism (18 outbreaks), 1 from food, and 3 from infant botulism. In addition, 4 *C. botulinum* type B strains from other countries (2 from United Kingdom, 1 from Switzerland, and 1 from Middle East,) were included (table 1).

**Table 1***Clostridium botulinum* Strains, Typing and Origin

Strain	Type/ Subtype	Botulinum Locus	ST	WGS	Botulism	Source of Strain Isolation	Year of Isolation
57	A1	ha	1		Cattle botulism	Cattle liver	1947
865	A1	ha	1		Cattle botulism	Cattle liver	1953
910	A1	ha	1		Cattle botulism	Cattle	1953
969	A1	ha	1		Avian botulism		1953
F18	A1	ha	1		Cattle botulism	Cattle liver	1953
F60	A1	ha	1		Cattle botulism	Cattle liver	1954
P179	A1	ha	1		unknown	Pike's gut	1953
P64	A1	ha	1		unknown	Eel's gut	1953
B697*	A1	ha	1		unknown	Catgut	1952
Legroux	A1	ha	1		Food-borne botulism	Garden pea	1947
PP	A1	ha	1		Food-borne botulism	Garden pea	1947
F14	A1	ha	38		Cattle botulism	Cattle liver	1953
424.86*	A1	ha	19		Cattle botulism	Cattle	1986
200-04	A1	orfx	37		Infant botulism	Patient's Feces	2004
207-04	A1	orfx	18		No botulism associated	Couscous	2004
Ba657*	Ba4	ha (orfx)	7		Infant botulism United States	Patient's Feces	1976
102-09	A1(B)	orfx (ha)	7		Infant botulism	Patient's Feces	2009
1134-11	A1(B)	orfx (ha)	7	x	Food-borne botulism	Green Olive Paste	2011
1161-11	A1(B)	orfx (ha)	7	x	Food-borne botulism	Gastric liquid	2011
1162-11	A1(B)	orfx (ha)	7	x	Food-borne botulism	Rectal swab	2011
1163-11	A1(B)	orfx (ha)	7	x	Food-borne botulism	Patient's Feces	2011
1193-11	A1(B)	orfx (ha)	7	x	Food-borne botulism	Gastric liquid	2011
1194-11	A1(B)	orfx (ha)	7	x	Food-borne botulism	Patient's Feces	2011
1195-11	A1(B)	orfx (ha)	7	x	Food-borne botulism	Patient's Feces	2011
1206-11	A1(B)	orfx (ha)	7	x	Food-borne botulism	Patient's Feces	2011
1209-11	A1(B)	orfx (ha)	7	x	Food-borne botulism	Patient's Feces	2011
654-12	A1(B)	orfx (ha)	7	x	Food-borne botulism	Green Olive Paste	2012
655-12	A1(B)	orfx (ha)	7	x	Food-borne botulism	Dried tomato Paste	2011
1151-11	A1(B)	orfx (ha)	7	x	Food-borne botulism	Patient's Feces	2011
186-13	A2	orfx	7	x	No botulism associated	Honey + Almond	2013
155-07	A1(B)	orfx (ha)	10		No botulism associated	Industrial Ready Meal	2007
714-12	A1(B)	orfx (ha)	10	x	Food-borne botulism	Home-made canned eggplant (imported from Morocco)	2012
88-12	A1(B)	orfx (ha)	15		Infant botulism	Patient's Feces	2012
88-05	A1(B)	orfx (ha)	39		Food-borne botulism	Ham	2005
158-08	A1(F)	orfx	18		Food-borne botulism	Home-made Jam Winter Squash	2008
260-07	A2	orfx	2		Infant botulism	Patient's Feces	2007
169-13	A2	orfx	22	x	Infant botulism	Patient's Feces	2013
224-13	A2	orfx	22	x	Infant botulism	Patient's Feces	2013
303-13	A2	orfx	22	x	Infant botulism	Patient's Feces	2013
308-13	A2	orfx	22	x	Infant botulism	Patient's Feces	2013
374-13	A2	orfx	22	x	Infant botulism	Patient's Feces	2013
398-13	A2	orfx	22	x	Infant botulism	Patient's Feces	2013
429-13	A2	orfx	26	x	Infant botulism	Patient's Feces	2013
389-06	A2	orfx	26		Infant botulism	Patient's Feces	2006
746-11	A2	orfx	26		Infant botulism	Patient's Feces	2011
181.02*	A2	orfx	40		Food-borne botulism	Gorgonzola sauce	2002
133-06	A2	orfx	41		Food-borne botulism	Home-made Terrine	2006
136-06	A2	orfx	42			Patient's Feces	2006
013-10	A2	orfx	43		Infant botulism	Patient's Feces	2010
969-10	A2	orfx	44	x	Food-borne botulism		2010

(continued)

Table 1 Continued

Strain	Type/ Subtype	Botulinum Locus	ST	WGS	Botulism	Source of Strain Isolation	Year of Isolation
						Salad with home-made canned French beans	
9336	A2	orfx	45		Food-borne botulism	Canned asparagus	1990
397-13	A2	orfx	82	x	Food-borne botulism (home-made canned asparagus suspected)	Patient's Feces	2013
301-13	A2b2	orfx (ha)	81	x	Food-borne botulism (home-made	Patient's Feces	2013
302-13	A2b2	orfx (ha)	81	x	canned French beans)	Patient's Feces	2013
1141-11	A5(B3')	ha	16	x	Food-borne botulism	Patient's Feces	2011
1430-11	A5(B2')	ha	46	x	Food-borne botulism	Industrial Ready Meal (Pasta carbonara)	2011
126.07*	A5(B2')	ha	47	x	Wound botulism	Wound	2007
148-08	A7	orfx	48	x	Food-borne botulism	Industrial Ready Meal (Enchiladas)	2008
217-12	A8	orfx	49	x	Food-borne botulism (Many gone off industrial meals consumed)	Patient's Feces	2012
NCTC7273*	B1	ha	34		Food-borne botulism United Kingdom	Beans	<1947
1837	B2	ha	13		Food-borne botulism	Ham	1958
2345	B2	ha	54		Food-borne botulism (Ham)	Ham	1961
F11	B2	ha	54		Cattle botulism	Cattle liver	1953
306-05	B2	ha	38		Food-borne botulism	Home-made Paté	2005
211-02*	B2	ha	38		No botulism associated	Tuna in oil	2002
1315-11	B2	ha	51		Food-borne botulism	Home-made canned spinach	2011
1329-11	B2	ha	51	x	Food-borne botulism	Patient's Feces	2011
012-10	B2	ha	52		Infant botulism	Patient's Feces	2010
1009-10	B2	ha	53		Food-borne botulism (Salad with home-made canned French beans)	Stomach Mucous membrane	2010
999-10	B2	ha	53		Food-borne botulism	Home-made canned asparagus	2010
277-00*	B2	ha	55	x	Food-borne botulism	Foodstuff	2000
5295	B2	ha	56		No botulism associated	Bell pepper	2005
580-86	B2	ha	57		Food-borne botulism	Industrial canned asparagus	1986
94-09	B2	ha	58		Food-borne botulism (home-made canned asparagus suspected)	Patient's Feces	2009
BL6*	B2	ha	59		No botulism associated	Foodstuff	1985
472-00	B3	ha	60		Food-borne botulism	Patient's Feces	2000
338-05	B5	ha	50	x	Infant botulism	Patient's Feces	2005
094-13	Bf2	ha (orfx)	14	x	Infant botulism	Patient's Feces	2013
161-08	Bf2	ha (orfx)	14		Food-borne botulism (home-made pumpkin jam)	Patient's Feces	2008
1135-11	Bf2	ha (orfx)	14	x	Food-borne botulism	Green Olive Paste	2011
1136-11	Bf2	ha (orfx)	14	x		Dried tomato Paste	2011
BL5*	B4	ha	nd		No botulism associated	Foodstuff	1985
13780	B4	ha	nd		Food-borne botulism (Ham)	Ham	2005
300-05	B4	ha	nd		Food-borne botulism (Ham)	Ham	2005
178-09	B4	ha	nd		Food-borne botulism (Ham)	Ham	2009
17837 <sup>a</sup>	B4	ha	nd		Food-borne botulism (Ham)	Ham	2010
1007-10	B4	ha	nd		Food-borne botulism (Ham)	Ham	2010
1012-10	B4	ha	b	x	Food-borne botulism (Ham)	Ham	2010
317-13	B4	ha	a	x	Food-borne botulism (Ham)	Ham	2013
815-12	B4	ha	a	x	Food-borne botulism (Ham)	Ham	2012
84-10	E12	orfx	c	x	Food-borne botulism (Ham)	Ham	2010
ATCC9009*	E1	orfx	nd		Collection strain	Unknown	
P34	E3	orfx	nd		Environnement	Perch intestine	1951
HV*	E3	orfx	nd		Environnement	Marine sediment	1953

(continued)

**Table 1** Continued

Strain	Type/ Subtype	Botulinum Locus	ST	WGS	Botulism	Source of Strain Isolation	Year of Isolation
Ped2*	E3	orfx	nd	Environnement		Marine sediment	1955
1781-3A	E3	orfx	nd	Animal botulism		Unknown	1957
HV2*	E3	orfx	nd	Environnement		Marine sediment	1953
188-09	E3	orfx	nd	Food-borne botulism (hot-smoked whitefish of Canadian origin)		Gastric liquid	2009
639-11 <sup>a</sup>	E5	orfx	nd	Intestinal colonization		Patient's Feces	2011

NOTE.—The colors indicate *C. botulinum* A strains belonging to the same ST. Vertical bars on the left side indicate that the strains were from a same outbreak.

\*All the strains have been isolated in France, except the strains noted with \*: B697 (Sweden), 424-86 (Brazil), 181-02, 126-07 and 211-02 (Switzerland), 277-00 (Iran) (Mazuet, Bouchier, et al. 2015), BL5 and BL6 (United Kingdom), HV and HV2 (United States, San Francisco, Mayer's collection), Ped2 (Greenland). Strains Ba657 (Hattheway et al. 1981), NCTC7273 (Bowmer 1963), and ATCC9009 were included as reference strains.

<sup>a</sup>Strain nonisolated. DNA sequences obtained by PCR with DNA extracted directly from samples and/or after enrichment culture of the samples.

WGS, whole genome sequencing; nd, not defined; ST, sequence type.

Botulism type E is very rare in France. A total of 9 strains were investigated including 1 reference strain, 5 historical strains originated from France, United States, and Greenland (Prévot's collection), and 3 from recent human botulism cases in France (2 from food-borne botulism and 1 from intestinal colonization) (table 1).

#### *Clostridium botulinum* Type A and Subtypes

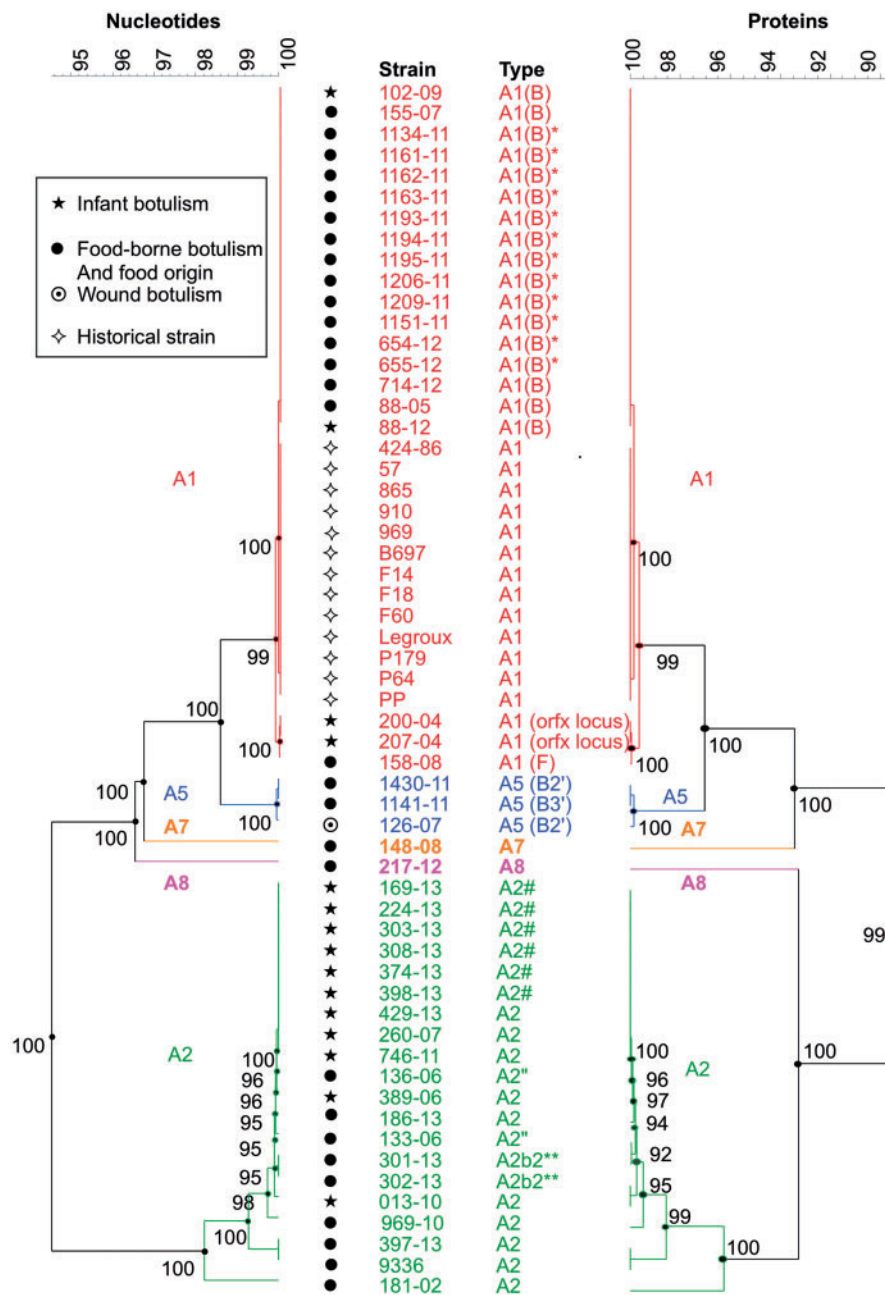
BoNT and ANTP genes were sequenced via specific PCR DNA amplification and sequencing and/or by WGS. Variations in *bont* genes were analyzed at the nucleotide and amino acid levels. Both nucleotide and amino acid sequence variations yielded similar level of subtype subdivision in each *C. botulinum* type (table 1 and fig. 1).

The 58 *C. botulinum* type A isolates were identified as subtype A1 (15 strains), A2 (18 strains), A1(B) (17 strains), A5(B') (3 strains), A1(F) (1 strain), A2(B) (2 strains) and 2 recently identified subtypes termed A7 (1 strain) and A8 (1 strain). No subtype A3 or A4 was detected. All the 12 historical strains belong to the subtype A1, whereas only 2 strains of 46 from the recent period were assigned to the subtype A1. However, these 2 strains contain *bont/A1* in OrfX locus, versus *ha-bont/A1* locus in the historical strains. In contrast, the strains isolated from food-borne botulism and food during the recent period were distributed in several subtypes (A1, A2, A5, A7, A8), and most of them (22 of 30) were bivalent strains [A1(B), A1(F), A2(B), A5(B')] (table 1 and fig. 1). The strains from a same outbreak shared the same *bont* type and subtype, except in two outbreaks where samples contained a mix of several *C. botulinum* types and subtypes, A1(B)/B5f2 and B/E12, respectively (table 1). *Clostridium botulinum* A1(B) was responsible for two large outbreaks of botulism in 2011 due to the consumption of commercial food with green olives (Pingeon et al. 2011), and for two other food-borne outbreaks subsequently to the ingestion of home-made ham and egg plant preparation, respectively (table 1). *Clostridium botulinum*

A2 was associated with several food-borne botulism outbreaks during the recent period (table 1). Notably, a severe outbreak including one decease and four patients hospitalized in intensive care unit with long-term mechanical ventilation was due to the ingestion of home-made canned beans contaminated with *C. botulinum* A2 in 2010 (Oriot et al. 2011) (table 1).

Two strains (1141-11 and 1430-11) from food-borne botulism contained a *bont* subtype A5 and a truncated *bontB* gene. The strain 1430-11 was responsible for a sporadic case of botulism subsequently to the consumption of a commercial ready-to-eat preparation (pasta carbonara), and the strain 1141-11 was isolated from stool of a food-borne botulism case of unknown origin (table 1). A third strain (126-07) A5 subtype was originated from wound botulism in Switzerland. BoNT/A5 sequences of strains 1141-11 and 1430-11 were identical at the nucleotide and amino acid level to that identified in an infant botulism case in California (Dover et al. 2010) and to that of a unknown origin strain which was characterized by the E Johnson's laboratory (Jacobson et al. 2011), whereas BoNT/A5 sequence of the strain 126-07 was similar to that of the strain isolated from wound botulism in United Kingdom (Carter et al. 2010) (table 2 and [supplementary fig. S1, Supplementary Material](#) online). The two strains 1141-11 and 1430-11 differed by their truncated *bont/B*. The strain 1430-11 contained a truncated *bont/B2* identical to the corresponding gene of United Kingdom and Swiss strain, whereas the strain 1141-11 harbored a truncated *bont/B3* gene related to that of the US strain ([supplementary fig. S1, Supplementary Material](#) online and table 2).

The two recently identified *bont* subtypes A7 and A8 showed 6–15% amino acid difference with the other known subtypes (table 3 and fig. 1). *Clostridium botulinum* A7 was isolated from an outbreak including two severe cases of botulism due the ingestion of a commercial ready-to-eat product containing chicken enchiladas (King 2008). The



**Fig. 1.**—Phylogenetic relatedness of BoNT/A nucleotide sequences and deduced proteins. The dendrograms were constructed using the UPGMA method. The genetic distances were computed by using the Kimura two-parameter model. The scale bar indicates similarity values. The numbers next to each node indicate the cophrenetic correlation. Evolutionary analyses were conducted in Bionumerics (V.6.6 Applied Maths). Strains noted \*, \*\*, # were isolated from a same botulism outbreak.

*C. botulinum* A8 strain was recovered in France in 2012 from stool of a patient who was living alone and who was used to consume out-of-date industrial food products and was similar to that described by (Kull et al. 2015). The source of the contamination was not identified.

The type A strains from infant botulism in France mainly belonged to the A2 subtype (11 out of 15 isolated strains), which was involved in 6 out of 10 type A infant botulism

outbreaks. Two infant botulism strains were identified as subtype A1(B) and one A1 in OrfX locus (table 1).

BoNT/A1 sequences of *C. botulinum* A1 (HA or OrfX locus), A1(B), A1(F) strains were highly conserved at the nucleotide and amino acid level, whereas BoNT/A2 sequences showed significant variations. However, BoNT/A2 sequences from strains of a same outbreak were identical (table 1 and fig. 1).

**Table 2**  
*Clostridium botulinum* A5 Strains

Strain	Collection	Origin of Botulism	Location	ST	Year	Neurotoxin Gene Cluster
A661222	E. Johnson	Unknown	China	16	1981	A5 (B3')
1141-11	IP	Food-borne	France	16	2011	A5 (B3')
1430-11	IP	Food-borne	France	46	2011	A5 (B2')
HO4402-065	M. Peck	Wound	United Kingdom	47	2004	A5 (B2')
126-07	IP	Wound	Switzerland	47	2007	A5 (B2')
IBCA94-0216	S. Arnon	Infant botulism	United States	nd		A5 (B3')

NOTE.—The colors indicate the strains with identical sequence type (ST). nd, not determined; IP, Institut Pasteur.

**Table 3**  
Nucleotide/Amino Acid Sequence Identities of *bont/A* Genes and Predicted Proteins of *Clostridium botulinum* A Subtypes

Subtype	A2 Kyoto F	A3 Loch Maree	A4 Ba 657	A5 H0 4402065	A6 CDC41370	A7 148.08 <sup>a</sup>	A8 217.12 <sup>a</sup>
A1 ATCC3502	95/90	92/85	94.5/89	99/97	98/96	97/94	97/93
A2		97/93	94/88	95/90	96/92	94.5/90	96/93.5
A3			92/84	93/85	93/86	92/85	94/88
A4				94/87	94/88	93/87	94/89
A5					98/95	97/94	97/93.5
A6						96/93	96/93
A7							96/91

NOTE.—<sup>a</sup>Recently identified subtype (King 2008; Kull et al. 2015).

### *Clostridium botulinum* Type B and Subtypes

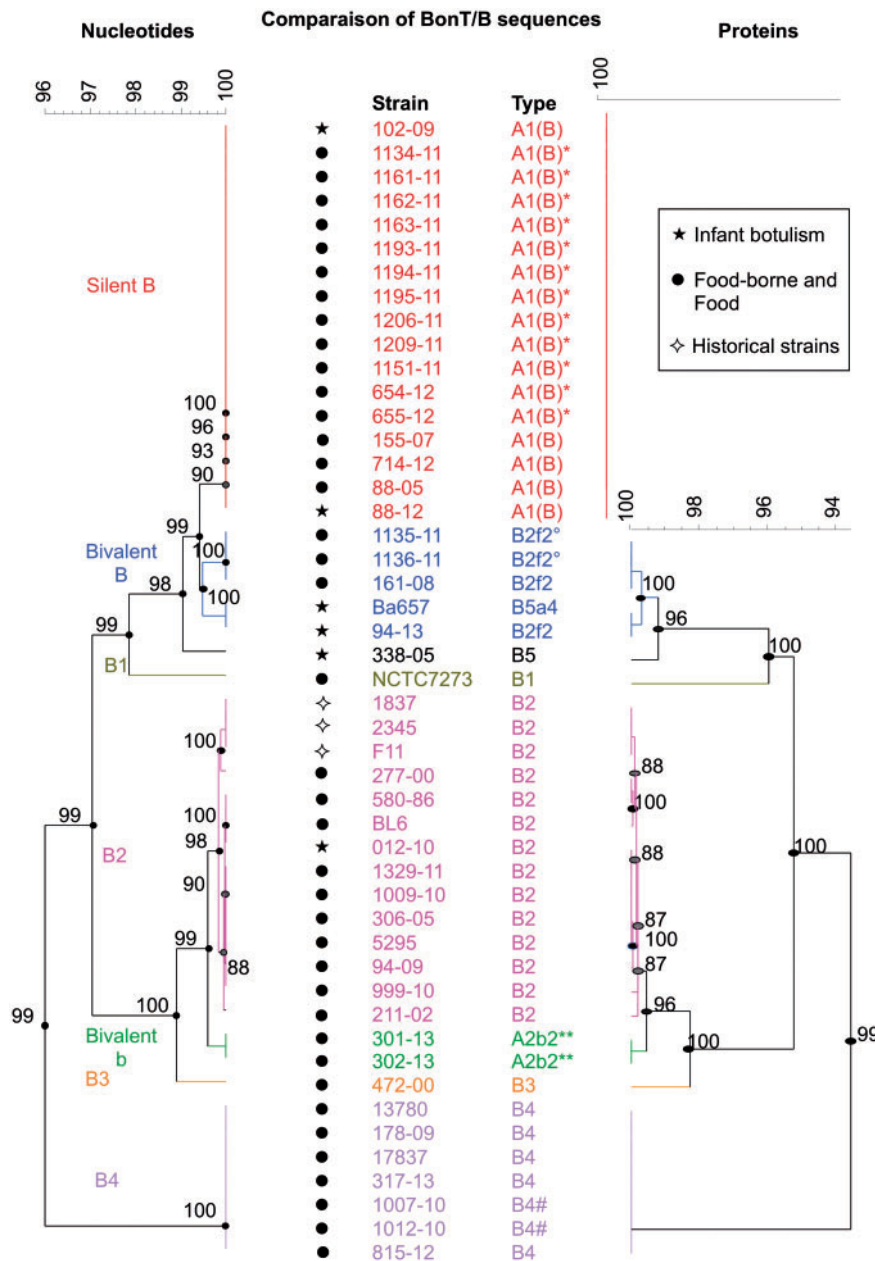
The 31 *C. botulinum* type B strains of this study consisted of 22 group I and 9 group II strains. Most of the group I *C. botulinum* B strains were subtype B2. Three historical *C. botulinum* B strains, two of them from food-borne botulism in France due to consumption of ham and one from cattle botulism, were subtype B2. In the recent period in France, 11 monovalent group I *C. botulinum* B strains have been isolated from food-borne botulism, and have been assigned to subtype B2, except one strain which was subtype B3. *Clostridium botulinum* B2 strains were most often (8 of 11) from canned vegetables (asparagus, spinach, bell pepper), and three from foods of animal origin (ham, homemade pâté, tuna). Two additional strains from food-borne botulism in United Kingdom and Iran (Pourshafie et al. 1998; Mazuet, Bouchier, et al. 2015), respectively, were also B2 subtype (table 1). Two monovalent group I *C. botulinum* B strains (B2 and B5) were isolated from infant botulism (table 1). An additional strain responsible for infant botulism was a bivalent B5f2 strain. BoNT/B2 sequences from all *C. botulinum* B2 strains from food-borne botulism, food and infant botulism were closely related at the nucleotide and amino acid levels (fig. 2).

All the 8 *C. botulinum* B strains which have been isolated from ham responsible for food-borne botulism during the

recent period, were nonproteolytic (Bnp or B4) strains of group II (table 1). An additional strain from food in United Kingdom was also a B4 subtype (table 1). BoNT/B4 sequences at the nucleotide and amino acid level were identical and were distantly related to those of group I *C. botulinum* B strains (fig. 2). BoNT/B4 showed 93–94.5% identity at the amino acid level with the other BoNT/B subtypes, whereas the relatedness between BoNT/B of group I strains ranged from 94% to 98% identity (table 4).

The *bont/B* sequence was the same in all *C. botulinum* A1(B) strains and contained a premature stop codon leading to a putative protein of only 127 amino acids. Therefore, the *bont/B* of the bivalent A1(B) strains cannot be significantly aligned with the other full length BoNT/B subtypes (fig. 2). Three *C. botulinum* bivalent B5f2 strains were isolated from food or stool of patient of a food-borne botulism outbreak due to the ingestion of vegetable preparations (olive, tomato, pumpkin) (table 1). It is noteworthy that two food samples containing the B5f2 strains (olive, tomato) were also contaminated with *C. botulinum* A1(B) (table 1). BoNT/B sequences of the *C. botulinum* B5f2 strains including an infant botulism strain were identical and closely related to the corresponding BoNT/B sequence of the bivalent B5a4 strain (fig. 2). But, they were distantly related to the BoNT/B2 sequences (figs. 1 and 2).





**Fig. 2.**—Phylogenetic relatedness of BoNT/B nucleotide sequences and deduced proteins. The dendrograms were constructed using the UPGMA method. The genetic distances were computed by using the Kimura two-parameter model. The scale bar indicates similarity values. The numbers next to each node indicate the cophrenetic correlation. Evolutionary analyses were conducted in Bionumerics (V.6.6 Applied Maths). Strains noted \*, \*\*, \*\*\*, # were isolated from a same botulism outbreak. For the A1(B) strains, the sequences of the truncated BoNT/B (nucleotides 1–384, 127 amino acids) were analyzed. These short BoNT/B sequences cannot be significantly compared with the other full length BoNT/B subtypes.

### *Clostridium botulinum* Type E and Subtypes

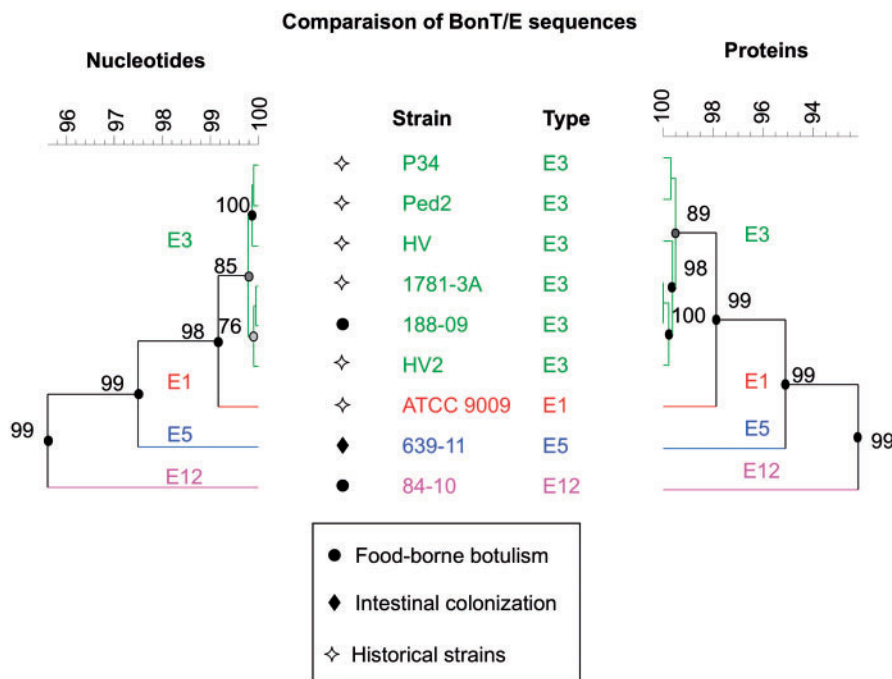
The historical *C. botulinum* type E strains (two from France, three from United States and Greenland environment samples) and one (188-09) of three from the recent period were subtype E3 (table 1 and fig. 3). The strain 188-09 was isolated from the gastric juice of a French patient out of three who

ingested vacuum packed hot-smoked whitefish of Canadian origin and processed in Finland (King et al. 2009). BoNT/E3 sequences of the historical strains and strain 188-09 were highly similar to that of the reference strain Alaska E43 (fig. 3 and [supplementary table S1, Supplementary Material online](#)).

**Table 4**

Nucleotide/Amino Acid Sequence Identities of *bont/B* Genes and Predicted Proteins of *Clostridium botulinum* B Subtypes

Subtype	B2 BL6	B3 472-00	Bnp (B4) 815-12	B5 338-05	B6 Osaka 05	B7 NCTC 3807	B8 Maehongson 2010
B1 NCTC 7273	98/96	98/96	96/93	97.5/95.5	98/96	98/95	98/95.5
B2		99/98	96/94	98/96	99/98	98/96	98/96
B3			97/94.5	98/96	98/97	98/96	98/96
B4				96/93	96/93.5	96/94	96/93
B5					97/95	97/94	97/94.5
B6						97/95	98/96
B7							97/95



**FIG. 3.**—Phylogenetic relatedness of BoNT/E nucleotide sequences and deduced proteins. The dendrograms were constructed using the UPGMA method. The genetic distances were computed by using the Kimura two-parameter model. The scale bar indicates similarity values. The numbers next to each node indicate the cophrenetic correlation. Evolutionary analyses were conducted in Bionumerics (V.6.6 Applied Maths). The strain ATCC9009 was included as reference strain subtype E1.

A recently identified *C. botulinum* E subtype, termed E12, was isolated from ham which was responsible for botulism in two persons. Ham sample contained both BoNT/B and BoNT/E but only a *C. botulinum* E strain (84-10) was isolated. BoNT/B and BoNT/E have been evidenced in the serum of the two patients (Mazuet, Sautereau, et al. 2015). BoNT/E12 from strain 84-10 showed 91–96% identity at the amino acid level with the other BoNT/E subtypes (supplementary table S1, Supplementary Material online).

The third botulism type E case was a botulism by intestinal colonization in a 10-year-old children having a Meckel’s diverticulum. The boy had a history of chronic constipation and diplopia two years before an episode of intense asthenia,

dry mouth, dysphonia followed by a cardio-respiratory arrest which was controlled by intubation and mechanical ventilation. *Bont/E* was PCR amplified (referred as 639-11, table 1) from stool, and matched with the subtype E5 (fig. 3), indicating the presence of neurotoxicogenic *C. butyricum* rather than *C. botulinum* E. However, isolation of a viable neurotoxicogenic *Clostridium* strain was unsuccessful. BoNT was evidenced neither in the serum nor in stool of the patient.

#### Diversity of the BoNT-Associated Nontoxic Proteins

Genomic comparison of the *antp* genes has been performed in the 40 strains for which WGS has been performed (table 1).

NTNH sequences clustered according to the locus type A, B, E or F (supplementary fig. S2, Supplementary Material online). In each locus type, NTNH sequence variations reflected the BoNT subtypes.

HA33 sequences showed variations also related to BoNT subtypes (supplementary fig. S2, Supplementary Material online). Phylogenetic analysis of HA33 nucleotide and amino acid sequences showed the same clade division of strains, except that the two *C. botulinum* A5 strains 1141-11 and 1430-11 were closely associated with *C. botulinum* A2b2 strains at the nucleotide level, and with *C. botulinum* B2 strains at the amino acid level (supplementary fig. S3, Supplementary Material online). In bivalent strains, *ha33* is located in the *bont/B* locus and HA33 sequence variations correlated with *bont/B* subtypes. However, the two *C. botulinum* A5 contained the same HA33 sequence, whereas *bont/B* subtypes were different, B3' and B2' (supplementary fig. S1, Supplementary Material online).

Phylogenetic analysis of HA17 and HA70 showed the same distribution of variants according to nucleotide and amino acid sequences, and that each clade corresponded to distinct *bont* subtype (supplementary figs. S4 and S5, Supplementary Material online). However, HA17 sequences were conserved in subtypes A1(B) and Bf2, whereas HA70 sequences were phylogenetically separated in these two subtypes (supplementary figs. S4 and S5, Supplementary Material online).

OrfXs showed variations at the nucleotide and amino acid levels which corresponded to the distinct *bont* subtypes A1(B), A2b2, Bf2, A2, A7 and A8. However, albeit OrfX variants clustered with individual *bont* subtypes, OrfX heterogeneity was observed in *C. botulinum* A2 and reflected the MLST diversity. OrfXs from *C. botulinum* E12 (strain 84-10) were distantly related to OrfX sequences of group I strains (supplementary figs. S6–S8, Supplementary Material online). OrfX2 is more conserved than OrfX1 and OrfX3 and shows lower phylogenetic difference than the two other OrfXs (supplementary figs. S6–S8, Supplementary Material online).

P47 sequences clustered with *bont* types and subtypes and showed no variation inside each *bont* subtype except in *C. botulinum* A2 strains where two P47 subgroups could be distinguished (supplementary fig. S9, Supplementary Material online). BotR also showed genetic variations at the nucleotide and amino acid level which clustered with the *bont* subtypes (supplementary fig. S10, Supplementary Material online).

### Genetic Diversity of Group I Strains

MLST analysis based on seven housekeeping genes have been found useful to elucidate phylogenetic lineages in group I *C. botulinum* (Jacobson et al. 2008; Luquez et al. 2012; Raphael et al. 2014). We used the MLST method developed by Jacobson et al. (2008) and the CDC data bank of *C. botulinum* MLST profiles. MLST based on PCR amplification and sequencing as well as on WGS was performed in 81 strains of

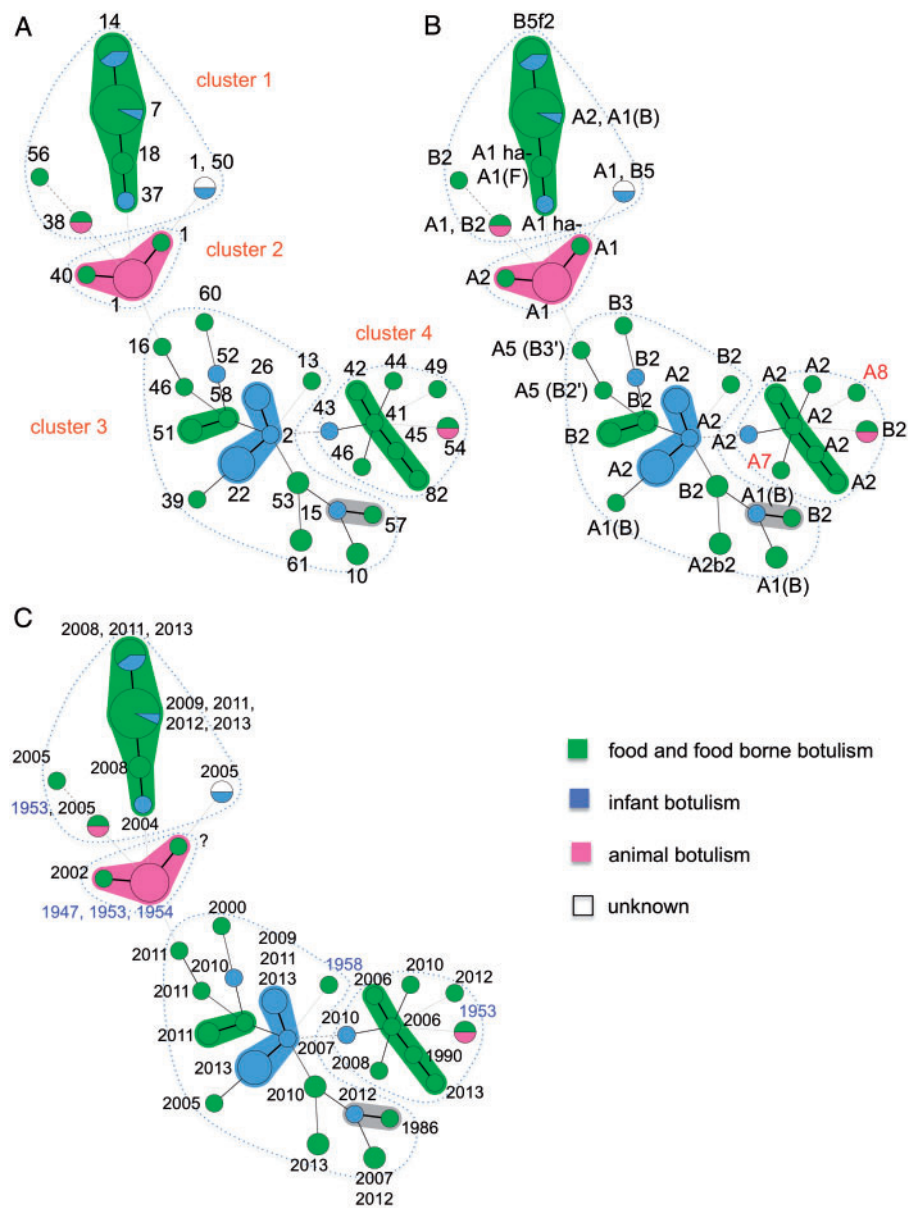
group I [59 *C. botulinum* A and A(B), and 22 *C. botulinum* B, Ba, and Bf] (table 1). MLST analysis revealed an extreme diversity of group I *C. botulinum* strains involved in human botulism in France. Out of 81 group I strains, 41 STs were identified, 16 matching with previously reported STs in the CDC data bank and 25 being novel STs (supplementary table S2, Supplementary Material online). Strains isolated from the same botulism outbreak shared identical ST (table 1) except in one outbreak where the strain isolated from food was a *C. botulinum* A2 ST41 and that from patient's stool was *C. botulinum* A2 ST42. However, two outbreaks in two distant places in France shared the same type of strains *C. botulinum* B2 ST53 (table 1). Only a low number of strains from different origin retained a conserved MLST profile, notably *C. botulinum* Bf2 ST14 was found in two independent food-borne outbreaks and one infant botulism case, and *C. botulinum* B2 ST38 and A1(B) ST10 were isolated in two independent foods and food-borne outbreaks, respectively (table 1).

Most historical *C. botulinum* A strains were assigned to ST1 (9 out of 10) and were mainly from animal origin. *Clostridium botulinum* A strains from the recent period showed diverse STs (25 profiles) distinct from ST1. The two *C. botulinum* A5 strains isolated in France from food-borne botulism belonged to distinct MLST profiles (table 1). The strain 1141-11 shared the same ST with that of the strain described by Johnson et al. (2011) evoking a possible common origin, whereas the strain 1430-11 showed a unique ST. The *C. botulinum* type A5 strain (126.07) isolated from wound botulism in Switzerland and that described by Carter et al. from wound botulism in United Kingdom which likely originated from Afghanistan (Carter et al. 2011) showed identical ST (table 2) suggesting a common origin, which remains to be determined.

*Clostridium botulinum* type B from group I also belonged to diverse STs (19 strains, 11 STs). *Clostridium botulinum* B2 strains or bivalent strains from France were in a distinct ST than those of strains from other countries such as *C. botulinum* B1 NCTC7273 or strains from United Kingdom (BL6) or Iran (277.00) (table 1). The four *C. botulinum* B5f2 strains from three different outbreaks belonged to the same ST (ST14), and two historical strains shared identical ST different from those of the recent strains (table 1).

*Clostridium botulinum* strains from infant botulism were also diverse, 10 STs were identified in strains from 12 infant botulism cases. A same strain, *C. botulinum* A2 ST26, was recovered in three infants at different period (2006, 2011, and 2013) and in distinct locations in France (Paris, Grenoble, Toulouse), whereas in the other infant botulism cases a unique ST was identified. In most cases, STs of strains from food-borne botulism were different from those of infant botulism supporting a distinct source of contamination in the two forms of botulism. However, two STs (ST7 and ST14) were shared by strains from both botulism forms (table 1).

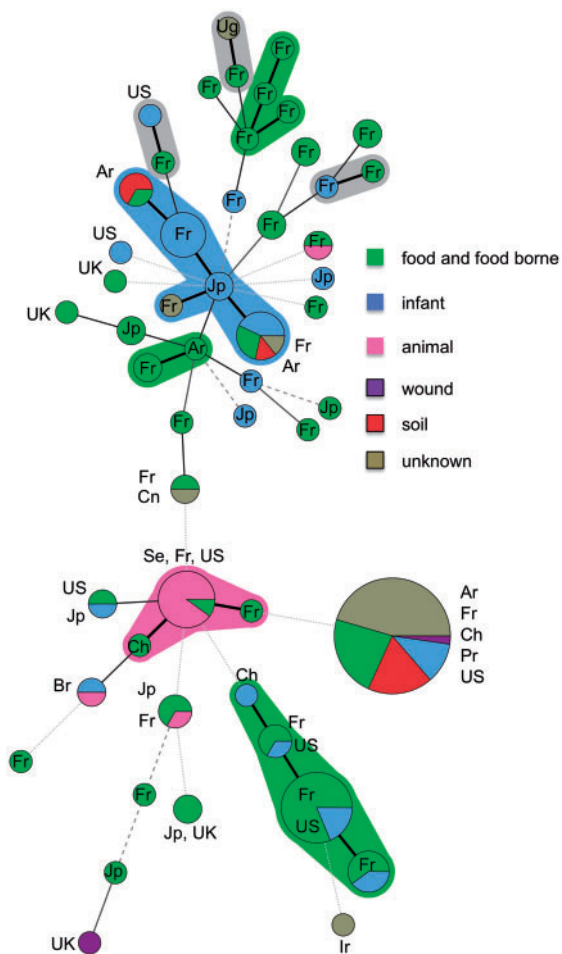
Relations within STs by clustering analysis using the maximum parsimony tree constructing method are shown in fig. 4.



**Fig. 4.**—Genetic relationship based on MLST analysis in 74 group I *Clostridium botulinum* isolates from France. The circles represent the different STs, and the sizes correspond to the number of strains with a particular genotype. Circles are colored according to the origin of strain (food and food-borne botulism, infant botulism, animal botulism or unknown origin). Similar types are connected by lines and halos depict MLST complexes. Heavy lines connecting two circles denote single locus variants, thin lines connect double locus variants, dotted lines show triple locus variants. Evolutionary analyses were conducted in Bionumerics (V.6.6 Applied Maths). (A) Strain relatedness of 74 group I *C. botulinum* isolates. (B) Strain relatedness according to types and subtypes of the *C. botulinum* isolates. (C) Strain relatedness according to the date of isolation. Historical strains (1947–1961) are indicated by the year written in blue.

STs of 74 group I isolates from France are distributed into 5 complexes and 19 singletons organized in 4 large clusters (fig. 4A). Figure 4B shows that the *C. botulinum* A and B subtypes are diversely spread in STs. Cluster 1 contained mainly strains from food-borne botulism, and cluster 2 mostly consisted of strains from animal origin. However, strains from food-borne botulism were spread into the four

clusters. *Clostridium botulinum* strains from infant botulism were mainly assigned to cluster 3, but some of them belonged to cluster 1 and 4. The historical strains (isolation between 1947 and 1961) were disseminated into different clusters, notably cluster 2, and the strains from the recent period showed a wider ST distribution. Thereby, *C. botulinum* strains did not evolved from a single lineage but from multiple



**FIG. 5.**—Genetic relationship based on MLST analysis in 151 group I *Clostridium botulinum* isolates from France and worldwide strains. The circles represent the different STs, and the sizes correspond to the number of strains with a particular genotype. Circles are colored according to the origin of strain (food and food-borne botulism, infant botulism, animal botulism, wound botulism, soil or unknown origin). Similar types are connected by lines and halos depict MLST complexes. Heavy lines connecting two circles denote single locus variants, thin lines connect double locus variants, dotted lines show triple locus variants. Evolutionary analyses were conducted in Bionumerics (V.6.6 Applied Maths). The 151 strains include 74 French isolates (fig. 4), 7 strains from Switzerland, Brazil, Sweden, Iran, and United Kingdom (table 1) and 2 reference strains (Ba657 and NCTC7273, from United States and United Kingdom, respectively) MLST sequences of which have been determined in this study, as well as 68 group I *C. botulinum* sequences available in GenBank. Ar, Argentina (40); Br, Brazil (1); Cn, China (1); Fr, France (74); Jp, Japan (10); Ir, Iran (1); Pr, Puerto Rico (1); Se, Sweden (1); Ch, Switzerland (3); UK, United Kingdom (4); Ug, Uganda (1); US, United States of America (14). Numbers in brackets indicate the numbers of strains.

phylogenetic pathways. Comparison of French isolates with group I strains from other countries available on databases was performed by phylogenetic MLST analysis (fig. 5). *Clostridium botulinum* strains from France as well as from

other countries were distributed in the four clusters, and no unique evolutionary lineages could be delineated for strains from a same location.

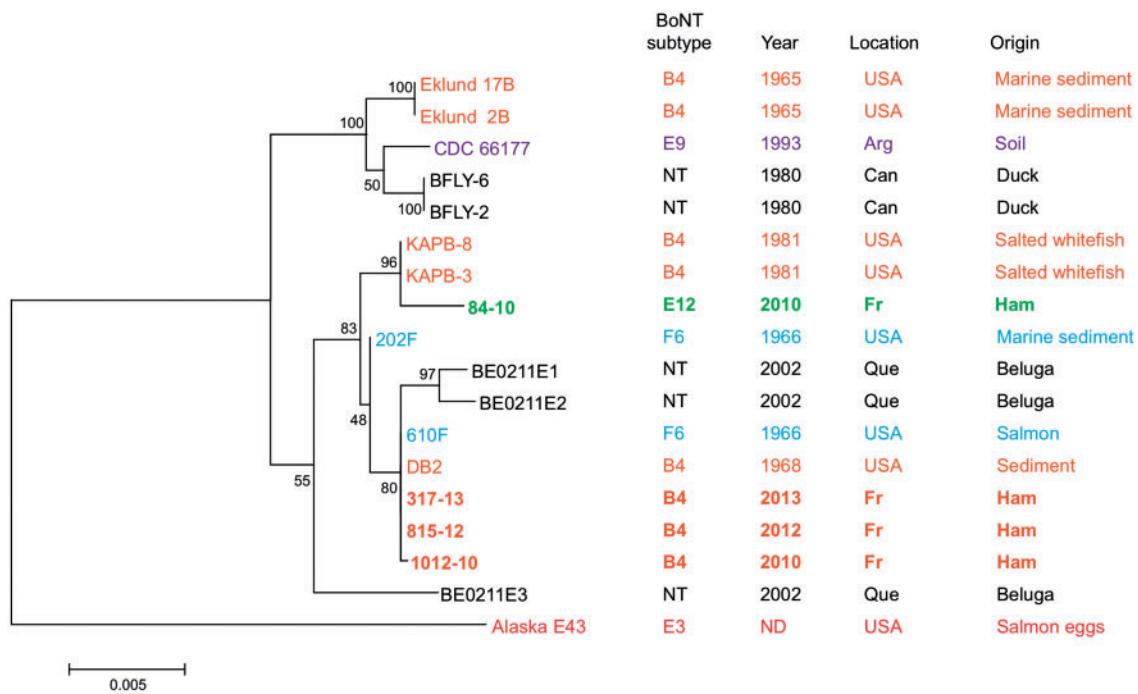
### MLST Analysis of Group II Strains

MLST analysis has been determined from WGS of 4 nonproteolytic *C. botulinum* group II strains (table 1). As the house-keeping gene sequences of group I strains cannot be used in group II strains due to sequence diversity, we exploited the sequences of 12 house-keeping genes as previously defined (Macdonald et al. 2011; Weedmark et al. 2014). Dendrogram of 12 concatenated house-keeping gene sequences from the four strains of this study and group II strains of databases is shown in fig. 6. The three *C. botulinum* B4 strains isolated from ham in France belonged to the same phylogenetic branch together with another B4 strain from United States, a *C. botulinum* F6 strain and two *C. botulinum* E strains from North America (fig. 6). The *C. botulinum* E12 strain was on a distinct phylogenetic lineage (fig. 6).

### Discussion

Among the 99 strains of group I and II investigated in this study, 20 were historical strains (1947–1961) (12 *C. botulinum* A, 3 *C. botulinum* B, and 5 *C. botulinum* E) and 69 (43 *C. botulinum* A, 23 *C. botulinum* B, and 3 *C. botulinum* E) were isolated during the recent period (1986–2013) in France from food or biological samples related to human botulism. Additional 3 *C. botulinum* A and 4 *C. botulinum* B strains originated from other countries in the recent period were included (table 1). The main finding is the broad diversity of the *C. botulinum* subtypes and genomes of strains from the recent period as monitored by botulinum locus gene sequencing and MLST analysis. Indeed, the *C. botulinum* A strains encompassed the subtypes A1 (*ha-bont/A1* and *orfX-bont/A1* locus), A1(B), A1(F), A2, A2b2, A5(B2') A5(B3'), as well as the recently identified A7 and A8 subtypes, and were distributed into 23 STs (table 1). In contrast, the historical *C. botulinum* A strains were more homogeneous. They all contained a *ha-bont/A1* locus and mainly belonged to ST1. However, the low number of historical strains and their origin mainly from animal or environment prevented precise evolution analyses of *C. botulinum* A strains involved in human botulism in France. As human botulism type A was rare in France in the past, the recent and outbreaks with diverse *C. botulinum* A STs mainly resulted from increased importation of food preparations or food products including spices subsequently transformed and commercialized in France. However, food-borne botulism outbreaks with home-made preparations from local products and infant botulism rather reflected the prevalence and diversity of *C. botulinum* A in the environment in France which was underestimated until now.

Bivalent *C. botulinum* A strains, mainly A1(B), were frequently isolated from food-borne botulism and foods



**Fig. 6.**—MLST molecular phylogenetic analysis for *Clostridium botulinum* group II isolates. Concatenated nucleotide sequences of 12 MLST loci were aligned with Muscle (default parameters) and the phylogenetic tree reconstructed by using the Maximum Likelihood method based on the Kimura 2-parameter model (Kimura 1980). About 1,000 bootstrap experiments were performed to assess the robustness of the topology and percents are indicated in nodes. The scale bar indicates the genetic distance (number of nucleotide substitutions per site). Evolutionary analyses were conducted in MEGA6 (Tamura et al. 2013). Strains described in this study are in bold. BoNT subtype, year, location, sample type and origin are indicated as follows: orange, BoNT/B4; turquoise, BoNT/F6; red, BoNT/E3; purple, BoNT/E9; green, BoNT/E12; and black, nontoxicogenic (NT). Abbreviations: ND, no data; Arg, Argentina; Can, Canada; Que, Quebec; Env, environmental. The new *pta* allele identified for the strain 1012-10 has been deposited in GenBank (KU 351681). The new alleles (GenBank KM370304 to KM370318) for 15 MLST loci of the strain 84-10 are available on supplementary table of Mazuet, Sautereau, et al. (2015).

[15 A1(B), 5 other bivalent strains of 32 *C. botulinum* A from food-borne botulism and foods]. In United States, bivalent A1(B) strains were the most frequently identified strains. Indeed, between 2010 and 2013, 86% of 47 *C. botulinum* A strains isolated from food or food-borne botulism samples were highly genetically related A1(B) subtypes (Raphael et al. 2014). In contrast, *C. botulinum* A1(B) strains from Japan were found to cluster in two lineages (Kenri et al. 2014). *Clostridium botulinum* A1(B) was identified in 6 outbreaks in France (2 infant botulism, and 4 food and food-borne botulism) and belonged to 4 STs, two of them being common to two outbreaks. Indeed, an industrial ready meal and an eggplant preparation from Morocco contained an identical *C. botulinum* A1(B) ST10. A common ST (ST7) was also found between an infant botulism case and one large outbreak of food-borne botulism due to the consumption of green olives and dried tomatoes (table 1). Bivalent A1(B) strains from United States belong to ST4, whereas those of France exhibited distinct STs suggesting different contamination sources in the two countries.

*Clostridium botulinum* A2 from various origin have been found to retain highly conserved (99.9–100% identity)

*bont/A2* and to be divided into 6 STs (ST2 being the most frequent, and then 7, 22, 26, 27 and 28) (Luquez et al. 2012). In our study, 17 of 20 *C. botulinum* A2 strains shared conserved *bont/A2*, and three showed more divergent sequences. BoNT/A2 from two strains exhibited 99.1% identity and one strain 97.7% identity with the other BoNT/A2 sequences at the amino acid level (fig. 1). The genetic background of *C. botulinum* A2 seems to be more variable than previously reported (Luquez et al. 2012). *Clostridium botulinum* A2 strains from 11 independent outbreaks were distributed into 10 STs. In one outbreak, two different STs (ST41 and 42) have been found in food and stool samples. Three STs (2, 22, and 26) isolated from infant botulism in France have also been found in patient and environment samples in other countries (Luquez et al. 2012), whereas 7 were newly described STs (table 1).

Two botulism cases due to *C. botulinum* A5 have been identified in France. One case was associated with the consumption of an industrial meal (pasta carbonara) and the strain was assigned to a unique ST (ST46) (table 2), whereas the origin of the other case, possibly home-made preparations, has not been identified. The strain of the second case

was identical (ST16) with a *C. botulinum* A5 strain from China characterized by Johnson (Jacobson et al. 2008, 2011). A common origin of these two strains is doubtful. The patients with *C. botulinum* A5 botulism complained about very long recovery (>2 years). This raises a possible long action of BoNT/A5 which has not been experimentally evidenced (Whitemarsh et al. 2013).

The origin of the botulism case with the recently identified *C. botulinum* type A8 in France was not identified. This strain shares identical BoNT sequence with the recently reported *C. botulinum* A8 in Germany in an old man who consumed home-made green beans. BoNT/A8 was found to exhibit reduced ganglioside binding and enzymatic activity resulting in a lower biological activity compared with BoNT/A1 (Kull et al. 2015). These findings showing that the genetic diversity of BoNTs might impact their functional activity and subsequently their clinical relevance, strengthen the importance of BoNT subtyping determination.

Most of group I *C. botulinum* B strains including 11 strains from food or food-borne botulism, one from infant botulism during the recent period as well as two historical strains, were subtype B2 (table 1). It was already reported that *C. botulinum* B1 was most often originated from United States and associated with vegetables, whereas *C. botulinum* B2 strains were frequent in Europe and associated with animals or meat (Hill et al. 2007). However, in our study *C. botulinum* B2 strains were not only associated with meat, but also with vegetables (table 1). Similar findings were reported in Italy (Franciosa et al. 2009). In Japan, *C. botulinum* B2 is prevalent and some strains, notably from infant botulism, are related to B2 strains from Europe on the basis of common MLVA (Umeda et al. 2013). In our study, *C. botulinum* B2 strains from 14 independent outbreaks or food were divided into 11 STs which were distinct from those described in Japan (Umeda et al. 2013). The numerous STs suggest multiple sources of *C. botulinum* B2 strains with distinct geographical localizations. Other *C. botulinum* B subtypes were rare. *Clostridium botulinum* B3 and B5 were identified in an infant botulism and food-borne botulism outbreak, respectively (table 1). *Clostridium botulinum* B3 and B5 are rarely reported, one B3 strain was described in United States and B5 strain in Japan (Hill et al. 2007; Kenri et al. 2014). In addition, bivalent *C. botulinum* B5f2 was involved in two food-borne outbreaks and one infant botulism case. All *C. botulinum* B5f2 strains shared the same ST (table 1).

*Clostridium botulinum* strains responsible for infant botulism in France were also genetically diverse as strains from food-borne botulism: 6 subtypes [A1, A1(B), A2, B2, B5, Bf2], and 10 STs in strains from 12 outbreaks. Although *bont* subtypes are common with those found in food-borne botulism strains, most of STs from infant botulism strains were distinct suggesting different sources of contamination between the two botulism forms. This might also indicate that *C. botulinum* from certain STs are more adapted to induce an

intestinal colonization. However, some STs such as ST7 and ST14 can be found in both botulism forms.

Genetic variations also concerned the genes of the non-toxic proteins of the botulinum clusters. More particularly, *ntnh* variations matched the *bont* type and subtype variants and might be used in the determination of *C. botulinum* types and subtypes. MLST analysis is a powerful discriminative method which allowed to differentiate almost all the group I strains of this study at the outbreak level and presents benefits for comparing with strains on databases from other laboratories as already reported (Jacobson et al. 2008; Umeda et al. 2009; Olsen et al. 2014; Raphael et al. 2014). The group I strains showed a high genetic diversity, and albeit they can be classified into four phylogenetic groups, no genetic lineages could be defined based on *bont* type and subtype, geographic location, date of strain isolation, or origin. These results support that the evolution of botulinum locus genes is independent of that of the core genome accounting of the diversity of *bont* type and subtype in strains with various STs (Luquez et al. 2012; Raphael et al. 2014; Weedmark et al. 2014; Hill et al. 2015; Smith, Hill, Raphael 2015; Williamson et al. 2016).

The group II *C. botulinum* B strains showed identical *bont* sequences assigned to B4 subtype (fig. 2). Group II *C. botulinum* B4 have been reported to form a homogeneous group with identical *bont* gene sequences and highly related genomic background (Stringer et al. 2013). All the eight French *C. botulinum* B4 strains have been isolated from ham or food-borne botulism due to ham consumption (table 1). This correlates with previous reports indicating that *C. botulinum* type B is a frequent inhabitant of the digestive tract of pigs (Dahlenborg et al. 2001; Myllykoski et al. 2006). *Clostridium botulinum* B of group II seems to be a preferred contaminant of pig meat, only three ham samples contained group I *C. botulinum* A or B strains [two B2 and one A1(B) subtypes] (table 1). WGS of three *C. botulinum* B4 strains showed that they contain identical botulinum locus, with 100% identity of *ha*, *botR* and *ntnh* sequences at the nucleotide and amino acid levels (supplementary figs. S2, S3, S4, S5, and S10, Supplementary Material online). In silico MLST analysis showed that these three strains shared a common genetic background identical to that of four other group II strains from environment or fish originated from North America but it is questionable that these strains have a common origin (fig. 6). Group II strains, notably *C. botulinum* E, are highly prevalent in marine sediments, fish and other seafood from Northern hemisphere areas including United States, Canada, Scandinavia and Russia, whereas the group II *C. botulinum* type B strains are associated with meat products and more rarely with fish (Lindström et al. 2006; Carter and Peck 2015). Albeit group II *C. botulinum* types B and E are highly related at the genomic level, they can be divided in two clades differing notably by genes involved in carbohydrate utilization or transport (Stringer et al. 2013). The different physiologic

properties likely account for the different habitats between nonproteolytic *C. botulinum* B and E.

*Clostridium botulinum* type E is rarely found in France. During the recent period, one botulism outbreak was associated with a hot-smoked fish of Canadian origin containing a *C. botulinum* type E3 strain, which is common in North America (Macdonald et al. 2011). A recently identified *C. botulinum* type E (E12) was isolated from ham sample. This unusual contamination of ham could result from the seasoning used to prepare this product (Mazuet, Sautereau, et al. 2015). Interestingly, MLST analysis showed that the *C. botulinum* E12 strain belonged to a distinct phylogenetic branch and that the most related strains were two strains isolated from salted whitefish (fig. 6) supporting an environmental origin probably from salt. A third botulism type E case was an intestinal colonization with a toxigenic *C. butyricum* E5 from unknown origin (table 1).

Altogether these results highlight the genetic diversity of *C. botulinum* strains isolated in France from human botulism. Comparison with the historical strains indicates that the *C. botulinum* strains of the recent period did not result from a single evolutionary lineage but from multiple and independent genetic rearrangements. Local evolution of strains and commercial exchanges of food products with other countries might account for the diversity of French *C. botulinum* strains. Recent investigations also show similar *C. botulinum* diversity in other countries such as in Italy (Giordani et al. 2015), Japan (Kenri et al. 2014), Australia (McCallum et al. 2015) and other parts in the world (reviewed in Carter and Peck 2015; Hill et al. 2015; Smith, Hill, Raphael 2015; Smith, Hill, Xie, et al. 2015). Thereby, the BoNT-producing clostridia share a high genetic plasticity the mechanisms of which including acquisition or loss of genetic materials by mobile genetic elements (plasmids, phages) and genetic rearrangements by insertion, recombination, mutation events are not yet fully understood. Genetic rearrangement also results from horizontal gene transfer between neurotoxicogenic *Clostridium* strains. Indeed, horizontal gene transfer of *bont* or the whole toxin gene cluster mediated by transposition through insertion sequence elements, exchange of plasmids or phages has already been documented in *C. botulinum* (Hill et al. 2007, 2009; Skarin and Segerman 2011; Smith, Hill, Xie, et al. 2015; Williamson et al. 2016). Identical botulinum gene clusters in strains with various genomic backgrounds and phylogenetic clusters as monitored by ST (table 1 and fig. 4) support horizontal gene transfer between strains from group I and II. Moreover, different *bont* loci in a same genomic background such as *bont/Ba4*, *bont/A1(B)*, and *bont/A2* loci in *C. botulinum* ST7 (table 1) further argues for horizontal gene transfer from distinct neurotoxicogenic strains in a same recipient strain. The mode of evolution of *C. botulinum* strains is still speculative. For example, the *bontA1-ha* locus seems to be the ancestor in group I *C. botulinum* strains, because all the historical strains contain this type of toxin gene cluster. The *bont/A1-orfX* and

*bont/A2-orfX* clusters are then observed in monovalent or bivalent isolates of a more recent period. The *bont/A2-orfX* locus is mainly found in strains of the phylogenetic cluster 4 and seems to have subsequently disseminated in strains of the other phylogenetic clusters (fig. 4) suggesting horizontal gene transfer and recombination events. Several methods of genetic analysis have been used to address the genetic diversity of *C. botulinum* strains. Among them, MLST appears to be a robust discriminatory method which correlates in strain clustering with the other methods such as MLVA and PFGE (Jacobson et al. 2008; Luquez et al. 2012; Macdonald et al. 2011; Umeda et al. 2013; Gonzalez-Escalona, Timme, et al. 2014; Olsen et al. 2014; Weedmark et al. 2014). However, MLST failed to distinguish *C. botulinum* type A(B) strains, which could be differentiated by SNP analysis (Raphael et al. 2014). Compared with the standard MLST protocol based on PCR product sequencing, WGS allows MLST but also additional and complementary genetic analysis such as SNP analysis and investigation of genetic rearrangement events.

## Supplementary Material

Supplementary tables S1 and S2 and figs. S1–S10 are available at *Genome Biology and Evolution* online (<http://www.gbe.oxfordjournals.org/>).

## Acknowledgments

We thank M. Tichit for technical assistance. Sequencing was performed at the Genomics Platform, member of “France Génomique” consortium (ANR10-INBS-09-08). This publication used the *C. botulinum* MLST website (<http://pubmlst.org/cbotulinum>, last accessed May 10, 2016) developed by K. Jolley, University of Oxford, developed and funded by the Wellcome Trust. This work was supported by Institut Pasteur and Institut national de Veille Sanitaire

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Associate editor: Howard Ochman