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REVISED

# DIVERSITY OF GROUP I AND II *CLOSTRIDIUM BOTULINUM* STRAINS FROM FRANCE INCLUDING RECENTLY IDENTIFIED SUBTYPES

Genome, Biology and Evolution

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**ABSTRACT**

In France, human botulism is mainly food-borne intoxication, whereas infant botulism is rare. A total of 99 group I and II *C. botulinum* strains including 59 type A (12 historical isolates (1947-1961), 43 from France (1986-2013), 3 from other countries and one collection strain), 31 type B (3 historical, 23 recent isolates, 4 from other countries and one collection strain), and 9 type E (5 historical, 3 isolates, and one collection strain) were investigated by botulinum locus gene sequencing and MLST 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. *C. 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 one recent strains were subtype E3. A subtype E12 was recently identified from an unusual ham contamination. *C. 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, multi-locus sequence typing, whole genome sequencing

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## 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 7 toxinotypes based on their antigenicity properties in neutralization assay (Barash and Arnon 2014; Dover, et al. 2014; Peck, et al. 2011; Popoff 1995). 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 to BoNT/E which leads to shorter duration symptoms and BoNT/B which is most often responsible for mild botulism illness (Eleopra, et al. 1998; Foran, et al. 2003; Keller 2006; Keller, et al. 1999; Meunier, et al. 2003; O'Sullivan, et al. 1999). 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 (Hughes, et al. 1981; Jenzer, et al. 1975; Merz, et al. 2003; Potulska-Chromik, et al. 2013; Sobel 2005). 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 counteracts 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,

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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, et al. 2014a).

BoNTs are synthesized by *Clostridium botulinum* and atypical strains of *Clostridium butyricum* and *Clostridium baratii*. The *bont* genes are clustered with the genes of BoNT associated non-toxic 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. *C. botulinum* strains are heterogeneous at the phenotypic and genetic levels, and are classified into 4 groups (I to IV), group IV being assigned to a distinct species, *Clostridium 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), multi-locus sequence typing (MLST), multi-locus variable number tandem repeat analysis (MLVA), and single nucleotide polymorphism (SNP) based on whole genome sequencing (review in (Brüggemann, et al. 2011; Carter and Peck 2015; Hill and Smith 2013; Popoff and Bouvet 2013; Smith, et al. 2015a). It is noteworthy that whole genome sequencing 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, et al. 2015b).

Human botulism is rare but recurrently identified in France. From 6 to 31 human cases of botulism (6 to 27 outbreaks) are reported every year in our country (Carlier, et al. 2007; Mazuet, et al. 2011; Mazuet, et al. 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

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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 one case, respectively, within the period 2004-2013 (Carlier, et al. 2007; King, et al. 2010; Mazuet, et al. 2011; Mazuet, et al. 2014). Infant botulism is prevalent in some countries such as the 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 to 9 cases in the other countries (Koepke, et al. 2008). Differences in the dissemination of neurotoxigenic *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 since 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, MD) and agar selective medium (Mazuet, et al. 2015b). *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 n° 2013-0116). The presence of corresponding

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*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 µl reaction buffer containing 200 ng DNA, 1 µM of each primer, 1.5 mM $MgCl_2$, 250 mM of each dNTP, 20 mM Tris-HCl, pH 8.4, 50 mM KCl and 1 u Taq Polymerase (In Vitrogen). 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.

**Whole genome sequencing**

Whole genome sequencing (WGS) 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 (MLST) 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. (Jacobson, et al. 2008). For some strains, nucleotide sequences of housekeeping genes were deduced from whole genome sequencing (Table 1). MLST data were submitted to the *C. botulinum* MLST database (http://pubmlst.org/cbotulinum/). 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

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submitted to the *C. botulinum* MLST database.

**Multilocus sequence typing analysis of group II strains.**

Multilocus sequence typing (MLST) 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). 1,000 boostrap 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 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, et al. 2015b) were KF929215 and KM370319, for *bontA7* (Becher, et al. 2007) JQ954969, and for *bont/A8* KF667385.

## 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 one 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 one from Sweden, one 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 one in

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Switzerland, 14 with infant botulism (9 cases, France), 3 from food (France), one from a wound botulism in Switzerland, and one 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 one from cattle botulism. A reference strain subtype B1 isolated in UK 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), one from food, and 3 from infant botulism. In addition, 4 *C. botulinum* type B strains from other countries (2 from UK, one from Switzerland, and one from Middle East,) were included (Table 1).

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

### *C. botulinum* type A and subtypes

BoNT and ANTP genes were sequenced via specific PCR DNA amplification and sequencing and/or by whole genome sequencing. 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, 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). *C. 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

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preparation, respectively (Table 1). *C. 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 UK (Carter, et al. 2010) (Table 2 and Sup. Fig. 1). 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 the UK and Swiss strain, whereas the strain 1141-11 harbored a truncated *bont/B3* gene related to that of the US strain (Sup Fig. 1 and Table 2).

The two recently identified *bont* subtypes A7 and A8 showed 6 to 15% amino acid difference with the other known subtypes (Table 3 and Fig. 1). *C. 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 *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

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showed significant variations. However, BoNT/A2 sequences from strains of a same outbreak were identical (Table 1 and Fig. 1).

### *C. 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. *C. 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 paté, tuna). Two additional strains from food borne botulism in UK and Iran (Mazuet, et al. 2015a; Pourshafie, et al. 1998), 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 non-proteolytic (Bnp or B4) strains of group II (Table 1). An additional strain from food in UK 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 can not 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 foodborne 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

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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 (Fig. 1 and 2).

### *C. botulinum* type E and subtypes

The historical *C. botulinum* type E strains (2 from France, 3 from USA 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, Sup Table S1).

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, et al. 2015b). BoNT/E12 from strain 84-10 showed 91 to 96% identity at the amino acid level with the other BoNT/E subtypes (Sup Table S1).

The third botulism type E case was a botulism by intestinal colonization in a 10 years 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 neurotoxigenic *C. butyricum* rather than *C. botulinum* E. However, isolation of a viable neurotoxigenic *Clostridium* strain was unsuccessful. BoNT was evidenced neither in the serum nor in stool of the patient.

### Diversity of the BoNT associated non-toxic proteins

Genomic comparison of the *antp* genes has been performed in the 40 strains for which whole genome sequencing has been performed (Table 1). NTNH sequences clustered according to the locus type A, B, E or F (Sup. Fig. 2). In each locus type, NTNH sequence variations reflected the BoNT subtypes.

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HA33 sequences showed variations also related to BoNT subtypes (Sup Fig. 2). 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 (Sup. Fig. 3). 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' (Sup. Fig. 1).

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 (Sup. Fig. 4 and 5). However, HA17 sequences were conserved in subtypes A1(B) and Bf2, whereas HA70 sequences were phylogenetically separated in these two subtypes (Sup. Fig. 4 and 5).

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 (Sup. Fig. 6, 7, and 8). OrfX2 is more conserved than OrfX1 and OrfX3 and shows lower phylogenetic difference than the two other OrfXs (Sup. Fig. 6, 7, and 8).

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 (Sup. Fig. 9). BotR also showed genetic variations at the nucleotide and amino acid level which clustered with the *bont* subtypes (Sup. Fig. 10).

**Genetic diversity of group I strains**

MLST analysis based on 7 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 (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 whole genome sequencing 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

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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 (Sup Table 2). 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. *C. 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. (Jacobson, 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 UK which likely originated from Afghanistan (Carter, et al. 2011) showed identical ST (Table 2) suggesting a common origin, which remains to be determined.

*C. botulinum* type B from group I also belonged to diverse STs (19 strains, 11 STs). *C. 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 UK (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).

*C. 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 3 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

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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. STs of 74 group I isolates from France are distributed into 5 complexes and 19 singletons organized in four large clusters (Fig. 4A). Fig. 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. *C. 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 phylogenetic pathways. Comparison of French isolates with group I strains from other countries available on databases was performed by phylogenetic MLST analysis (Fig. 5). *C. 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 whole genome sequencing of 4 non-proteolytic *C. botulinum* group II strains (Table 1). Since the house keeping gene sequences of group I strains can not been 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). Dendogram 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 USA, 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**

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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. Since 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 (15 A1(B), 5 other bivalent strains of 32 *C*. *botulinum* A from food borne botulism and foods). In the US, 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). *C*. *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

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green olives and dried tomatoes (Table 1). Bivalent A1(B) strains from the US belong to ST4, whereas those of France exhibited distinct STs suggesting different contamination sources in the two countries.

*C. 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). *C. 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. 2011; Jacobson, et al. 2008). A common origin of these two strains is doubtful. The patients with *C. botulinum* A5 botulism complained about very long recovery (more than two 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 to 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.

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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 the US 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. *C*. *botulinum* B3 and B5 were identified in an infant botulism and food borne botulism outbreak, respectively (Table 1). *C*. *botulinum* B3 and B5 are rarely reported, one B3 strain was described in US 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).

*C*. *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; Olsen, et al. 2014; Raphael, et al. 2014; Umeda, et al. 2009). The

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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 (Hill, et al. 2015; Luquez, et al. 2012; Raphael, et al. 2014; Smith, et al. 2015a; Weedmark, et al. 2014; 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 an homogeneous group with identical *bont* gene sequences and highly related genomic background (Stringer, et al. 2013). All the 8 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). *C*. *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). Whole genome sequencing of 3 *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 (Sup. Fig. 2, 3, 4, 5, and 10). *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 the USA, Canada, Scandinavia and Russia, whereas the group II *C*. *botulinum* type B strains are associated with meat products and more rarely with fish (Carter and Peck 2015; Lindstrôm, et al. 2006). 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 non-proteolytic *C*. *botulinum* B and E.

*C*. *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.

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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, et al. 2015b). 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, et al. 2015a; Smith, et al. 2015b). 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 neurotoxigenic *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; Hill, et al. 2009; Skarin and Segerman 2011; Smith, et al. 2015b; 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 neurotoxigenic 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, since all the historical strains contain this type of toxin gene cluster. The *bont*/A1-*orfX* and *bont*/*A2-orfX* clusters are then

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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 (Gonzalez-Escalona, et al. 2014b; Jacobson, et al. 2008; Luquez, et al. 2012; Macdonald, et al. 2011; Olsen, et al. 2014; Umeda, et al. 2013; 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 to 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.

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**LEGENDS OF FIGURES AND TABLES**

**Table 1. *C. botulinum* strains. Table 1**. *Clostridium botulinum* strains, typing and origin.
* 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 (Middle East), BL5 and BL6 (UK), Ba657 (USA), HV and HV2 (USA, San Francisco, Mayer's collection), Ped2 (Greenland)
# Strain non-isolated. DNA sequences obtained by PCR with DNA extracted directly from samples and/or after enrichment culture of the samples.
The colors indicate *C. botulinum* strains belonging to the same group I MLST profile.
a, b and c indicate *C. botulinum* strains belonging to the same group II MLST profile.
ST:MLST Type
WGS: Whole Genome Sequencing

**Table 2.** *C. botulinum* A5 strains. The colors indicate the strains with identical sequence type (ST).
nd, not determined

**Table 3.** Nucleotide/amino acid sequence identities of *bont/A* genes and predicted proteins of *C. botulinum* A subtypes.
* recently identified subtype (King 2008; Kull, et al. 2015).

**Table 4.** Nucleotide/amino acid sequence identities of *bont/B* genes and predicted proteins of *C. botulinum* B subtypes

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**Figure 1 Phylogenetic relatedness of BoNT/A nucleotide sequences and deduced proteins.** The dendograms 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.

**Figure 2. Phylogenetic relatedness of BoNT/B nucleotide sequences and deduced proteins.** The dendograms 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 to 384, 127 amino acids) were analyzed. These short BoNT/B sequences can not be significantly compared to the other full length BoNT/B subtypes.

**Figure 3. Phylogenetic relatedness of BoNT/E nucleotide sequences and deduced proteins.** The dendograms 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.

**Figure 4. Genetic relationship based on MLST analysis in 74 group I *C. 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).
MLST types of 74 group I *C. botulinum* isolates.

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(A) Strains relatedness of 74 group I *C. botulinum* isolates.

(B) Strains relatedness according to types and subtypes of the *C. botulinum* isolates

(C) Strains relatedness according to the date of isolation. Historical strains (1947-1961) are in blue.

**Figure 5. Genetic relationship based on MLST analysis in 151 group I *C. 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 UK (Table 1) and 2 reference strains (Ba657 and NCTC7273, from the U.S. and UK, 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.

**Figure 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). 1,000 boostrap experiments were performed to assess the robustness of the topology and percent 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, nontoxigenic (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

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alleles (GenBank KM370304 to KM370318) for 15 MLST loci of the strain 84-10 are available on Supplementary Table of (Mazuet, et al. 2015b).

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| Strain | Type/ Subtype | Botulinum locus | ST | WGS[a] | 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 USA | 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) | Green Olive Paste | 2011 |
| 1161-11 | A1(B) | orfx (ha) | 7 | x | | Gastric liquid | 2011 |
| 1162-11 | A1(B) | orfx (ha) | 7 | x | | Rectal swab | 2011 |
| 1163-11 | A1(B) | orfx (ha) | 7 | x | | Patient's Feces | 2011 |
| 1193-11 | A1(B) | orfx (ha) | 7 | x | | Gastric liquid | 2011 |
| 1194-11 | A1(B) | orfx (ha) | 7 | x | | Patient's Feces | 2011 |
| 1195-11 | A1(B) | orfx (ha) | 7 | x | | Patient's Feces | 2011 |
| 1206-11 | A1(B) | orfx (ha) | 7 | x | | Patient's Feces | 2011 |
| 1209-11 | A1(B) | orfx (ha) | 7 | x | | Patient's Feces | 2011 |

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|---|---|---|---|---|---|---|---|
| 654-12 | A1(B) | orfx (ha) | 7 | x | | Green Olive Paste | 2012 |
| 655-12 | A1(B) | orfx (ha) | 7 | x | | Dried tomato Paste | 2011 |
| 1151-11 | A1(B) | orfx (ha) | 7 | x | | 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 | | | |
| 303-13 | A2 | orfx | 22 | X | | | |
| 308-13 | A2 | orfx | 22 | X | | | |
| 374-13 | A2 | orfx | 22 | X | | | |
| 398-13 | A2 | orfx | 22 | X | | | |
| 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 | Salad with home made canned French beans | 2010 |
| 9336 | A2 | orfx | 45 | | Food-borne botulism | Canned asparagus | 1990 |
| 397-13 | A2 | orfx | 82 | x | Food-borne botulism (Home made | Patient's Feces | 2013 |

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| | | | | | | | |
|---|---|---|---|---|---|---|---|
| | | | | | canned asparagus) | | |
| 301-13 | A2b2 | orfx (ha) | 81 | x | Food-borne botulism (Home made canned French beans) | Patient's Feces | 2013 |
| 302-13 | A2b2 | orfx (ha) | 81 | x | | 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) | 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) | 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 UK | 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 | | 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) | 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) | Industrial canned asparagus | 1986 |

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| | | | | | | | |
|---|---|---|---|---|---|---|---|
| 94-09 | B2 | ha | 58 | | Food-borne botulism (Home made canned asparagus) | 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) | 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# | 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 |
| 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 |

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|---|---|---|---|---|---|---|---|
| 639-11# | E5 | orfx | nd | | Intestinal colonization | Patient's Feces | 2011 |

**Table 1**. *Clostridium botulinum* strains, typing and origin.

* 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, et al. 2015a), BL5 and BL6 (UK), HV and HV2 (USA, San Francisco, Mayer's collection), Ped2 (Greenland). Strains Ba657 (Hatheway, et al. 1981), NCTC7273 (Bowmer 1963), and ATCC9009 were included as reference strains.

# Strain non isolated. DNA sequences obtained by PCR with DNA extracted directly from samples and/or after enrichment culture of the samples.

[a] Whole genome sequencing (WGS).

nd, not defined.

ST, sequence type. The colors indicate *C. botulinum* A strains belonging to the same ST.

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| Strain | Collection | Origin of botulism | Location | ST | Year | Neurotoxin gene cluster |
|---|---|---|---|---|---|---|
| **A661222** | 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 | UK | 47 | 2004 | A5 (B2') |
| **126-07** | IP | Wound | Switzerland | 47 | 2007 | A5 (B2') |
| **IBCA94-0216** | S.Arnon | Infant botulism | USA | nd | | A5 (B3') |

**Table 2.** *C. botulinum* A5 strains. The colors indicate the strains with identical sequence type (ST).
nd, not determined

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| Subtype | A2 Kyoto F | A3 Loch Maree | A4 Ba 657 | A5 H0 4402065 | A6 CDC41370 | A7 148.08* | A8 217.12* |
|---|---|---|---|---|---|---|---|
| **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 |

**Table 3.** Nucleotide/amino acid sequence identities of *bont/A* genes and predicted proteins of *C. botulinum* A subtypes.

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* recently identified subtype (King 2008; Kull, et al. 2015).

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| Subtype | B2<br>BL6 | B3<br>472-00 | Bnp (B4)<br>815-12 | B5<br>338-05 | B6<br>Osaka 05 | B7<br>NCTC 3807 | B8<br>Maehongson 2010 |
|---|---|---|---|---|---|---|---|
| **B1**<br>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 |

**Table 4.** Nucleotide/amino acid sequence identities of *bont/B* genes and predicted proteins of *C. botulinum* B subtypes.

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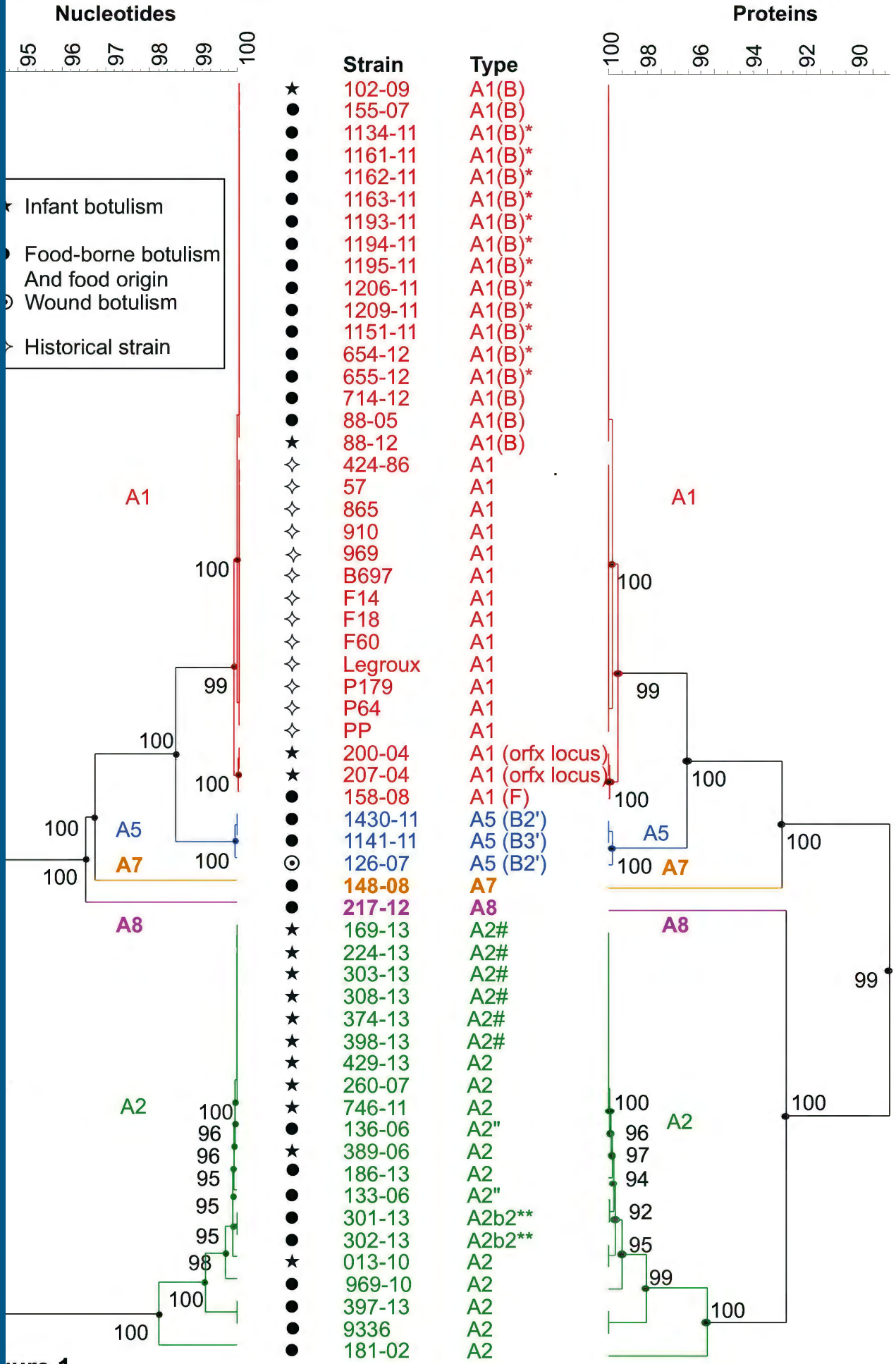
Nucleotides
95
96
97
98
99
100
Proteins
100
98
96
94
92
90
Strain
Type
Infant botulism
Food-borne botulism
And food origin
Wound botulism
Historical strain
102-09 A1(B)
155-07 A1(B)
1134-11 A1(B)*
1161-11 A1(B)*
1162-11 A1(B)*
1163-11 A1(B)*
1193-11 A1(B)*
1194-11 A1(B)*
1195-11 A1(B)*
1206-11 A1(B)*
1209-11 A1(B)*
1151-11 A1(B)*
654-12 A1(B)*
655-12 A1(B)*
714-12 A1(B)
88-05 A1(B)
88-12 A1(B)
424-86 A1
57 A1
865 A1
910 A1
969 A1
B697 A1
F14 A1
F18 A1
F60 A1
Legroux A1
P179 A1
P64 A1
PP A1
200-04 A1 (orfx locus)
207-04 A1 (orfx locus)
158-08 A1 (F)
1430-11 A5 (B2')
1141-11 A5 (B3')
126-07 A5 (B2')
148-08 A7
217-12 A8
169-13 A2#
224-13 A2#
303-13 A2#
308-13 A2#
374-13 A2#
398-13 A2#
429-13 A2
260-07 A2
746-11 A2
136-06 A2"
389-06 A2
186-13 A2
133-06 A2"
301-13 A2b2**
302-13 A2b2**
013-10 A2
969-10 A2
397-13 A2
9336 A2
181-02 A2
A1
A5
A7
A8
A2
100
99
96
95
98
97
94
92

Figure 1

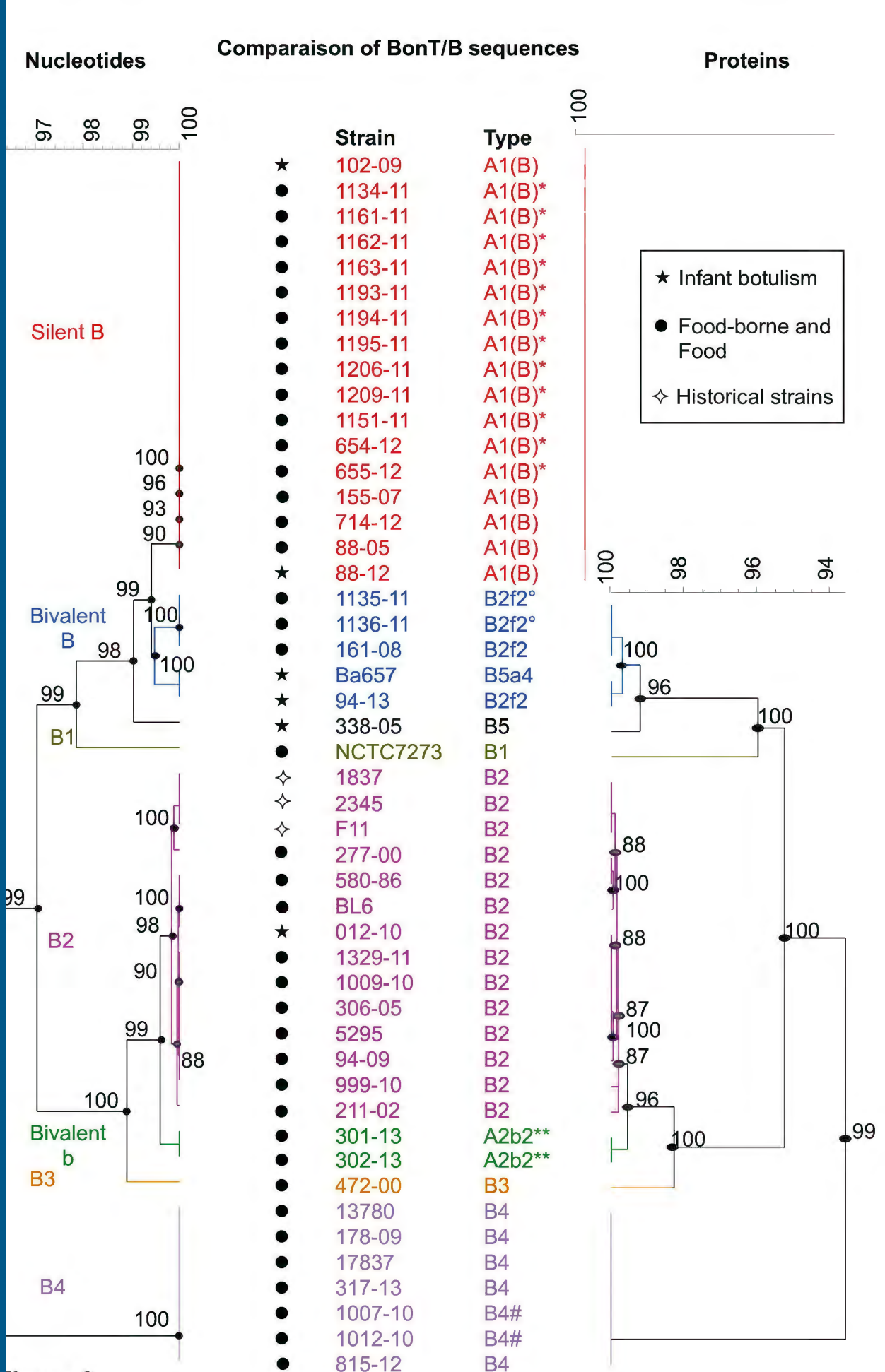
Comparaison of BonT/B sequences
Nucleotides
Proteins
Strain
Type
★ Infant botulism
● Food-borne and Food
✧ Historical strains
Silent B
Bivalent B
B1
B2
Bivalent b
B3
B4
★ 102-09 A1(B)
● 1134-11 A1(B)*
● 1161-11 A1(B)*
● 1162-11 A1(B)*
● 1163-11 A1(B)*
● 1193-11 A1(B)*
● 1194-11 A1(B)*
● 1195-11 A1(B)*
● 1206-11 A1(B)*
● 1209-11 A1(B)*
● 1151-11 A1(B)*
● 654-12 A1(B)*
● 655-12 A1(B)*
● 155-07 A1(B)
● 714-12 A1(B)
● 88-05 A1(B)
★ 88-12 A1(B)
● 1135-11 B2f2°
● 1136-11 B2f2°
● 161-08 B2f2
★ Ba657 B5a4
★ 94-13 B2f2
★ 338-05 B5
● NCTC7273 B1
✧ 1837 B2
✧ 2345 B2
✧ F11 B2
● 277-00 B2
● 580-86 B2
● BL6 B2
★ 012-10 B2
● 1329-11 B2
● 1009-10 B2
● 306-05 B2
● 5295 B2
● 94-09 B2
● 999-10 B2
● 211-02 B2
● 301-13 A2b2**
● 302-13 A2b2**
● 472-00 B3
● 13780 B4
● 178-09 B4
● 17837 B4
● 317-13 B4
● 1007-10 B4#
● 1012-10 B4#
● 815-12 B4

**Figure 2**

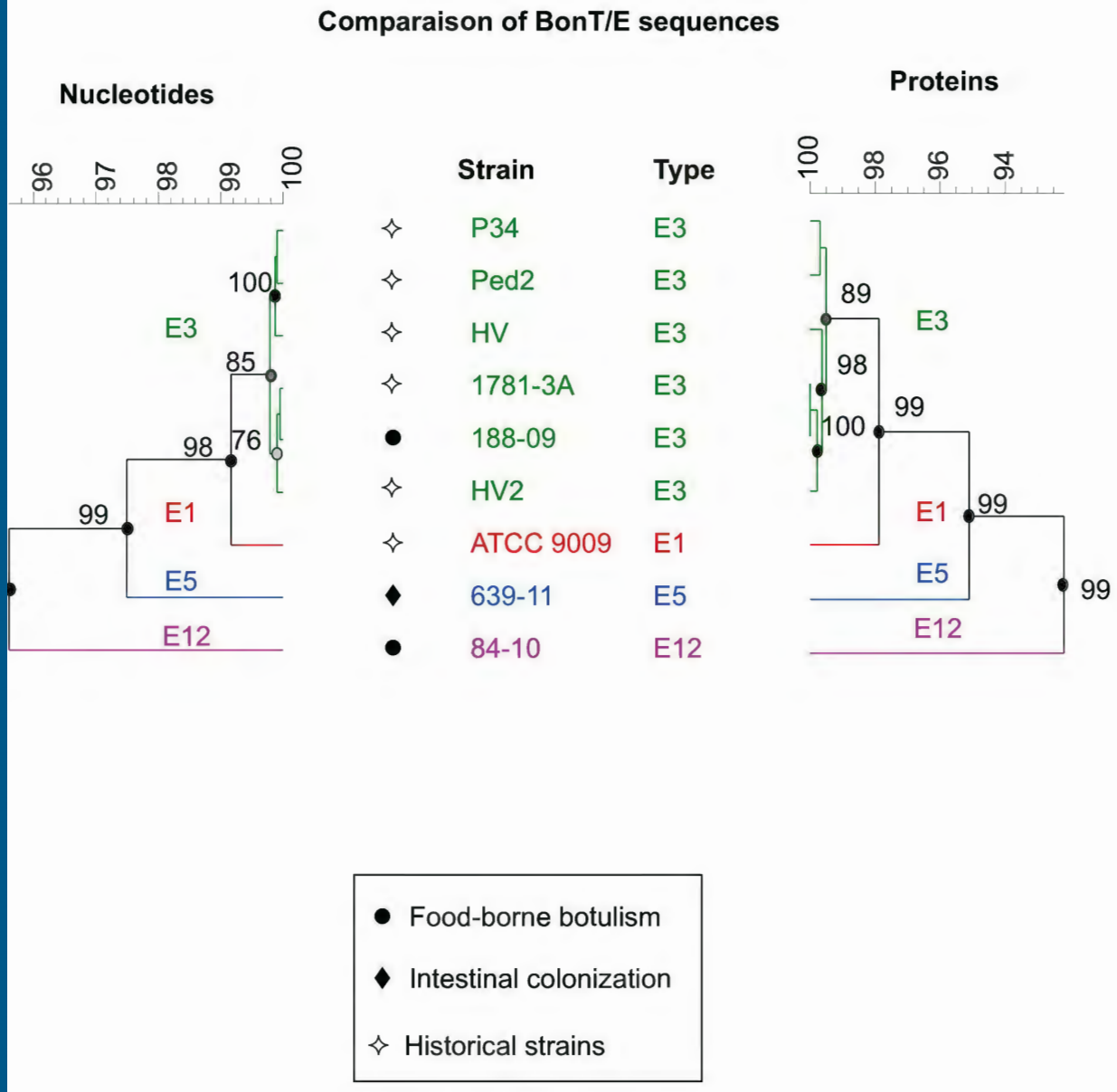
Comparaison of BonT/E sequences
Nucleotides
Proteins
Strain
Type
P34 E3
Ped2 E3
HV E3
1781-3A E3
188-09 E3
HV2 E3
ATCC 9009 E1
639-11 E5
84-10 E12
96
97
98
99
100
94
E3
E1
E5
E12
Food-borne botulism
Intestinal colonization
Historical strains

**Figure 3**

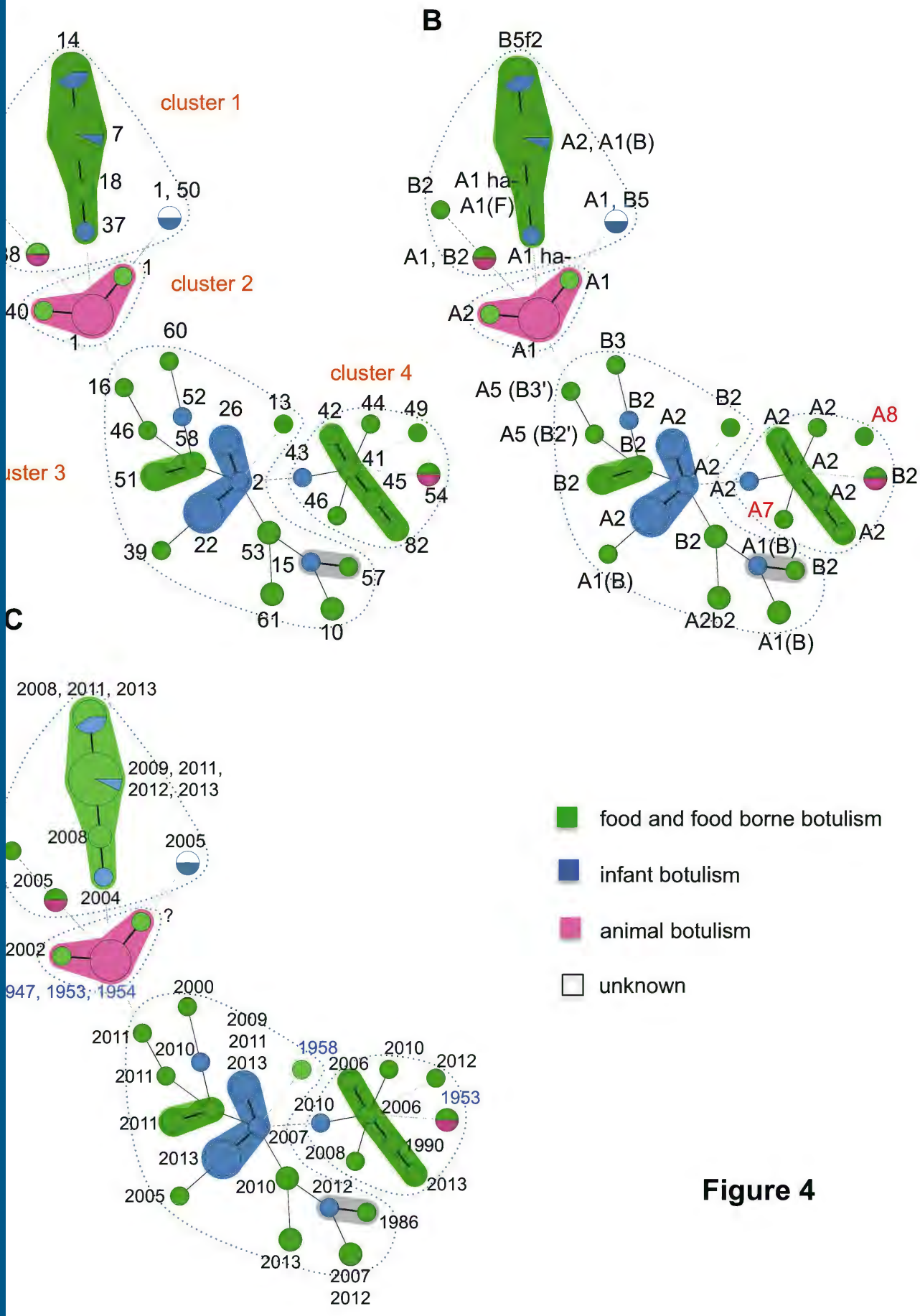
B
cluster 1
cluster 2
cluster 3
cluster 4
14
7
18
37
1, 50
1
60
16
52
46
58
26
13
51
2
42
44
49
43
41
45
54
46
82
39
22
53
15
57
61
10
B5f2
A2, A1(B)
B2
A1 ha-
A1(F)
A1, B5
A1, B2
A1 ha-
A1
A2
A1
B3
A5 (B3')
B2
B2
A5 (B2')
B2
A2
A2
A2
A2
A2
A8
B2
A2
A2
A2
A7
A2
B2
A2
B2
A1(B)
B2
A1(B)
A2b2
A1(B)
C
2008, 2011, 2013
2009, 2011,
2012, 2013
2008
2005
2005
2004
?
2002
947, 1953, 1954
2000
2009
2011
2011
2010
2011
1958
2006
2010
2012
2011
2013
2010
2006
1953
2011
2007
1990
2013
2008
2013
2010
2012
2005
2013
1986
2013
2007
2012
food and food borne botulism
infant botulism
animal botulism
unknown

**Figure 4**

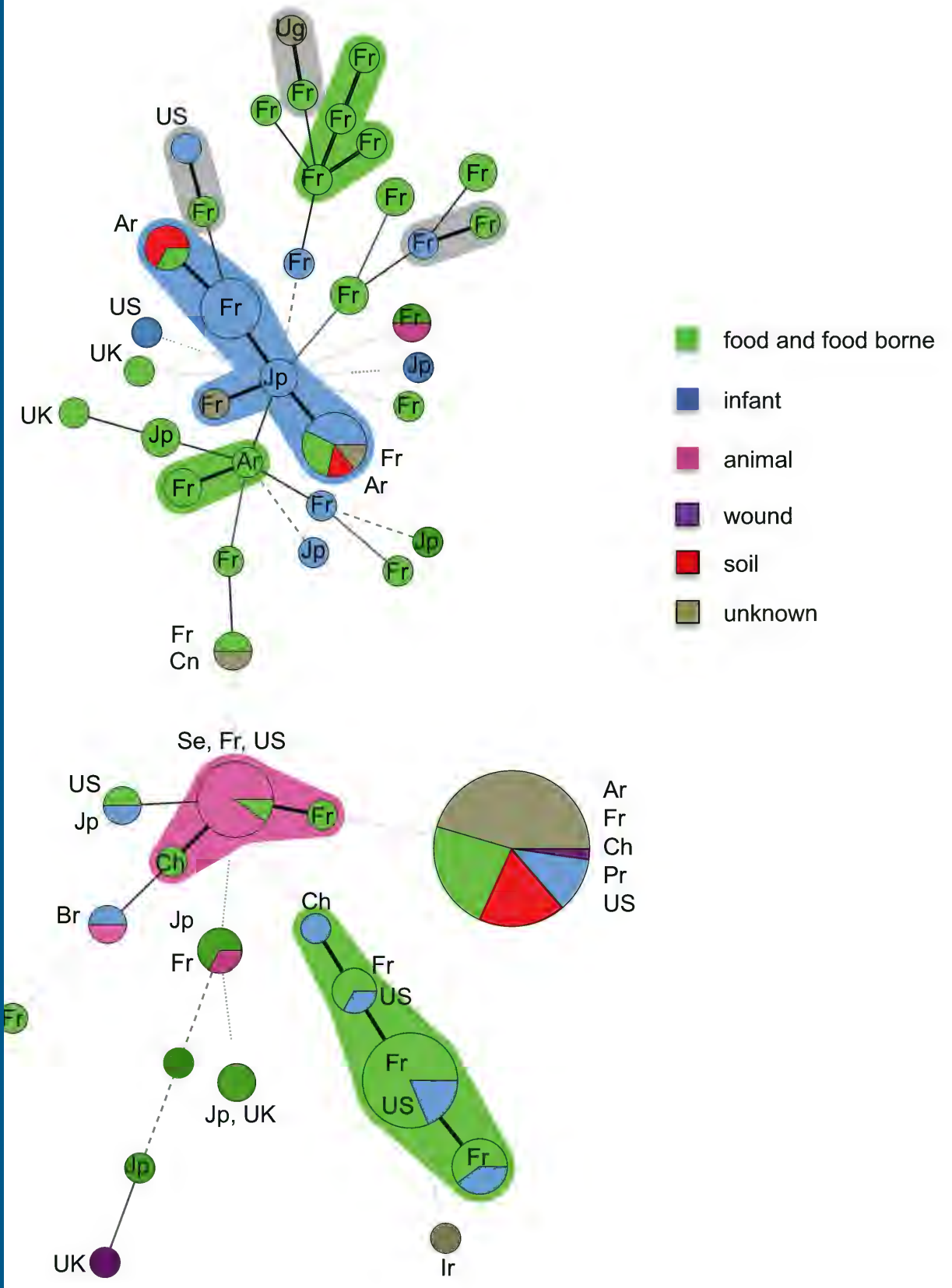
Ug
US
Ar
US
UK
UK
Jp
Ar
Fr
Ar
Jp
Fr
Cn
food and food borne
infant
animal
wound
soil
unknown
Se, Fr, US
US
Jp
Ch
Br
Jp
Fr
Jp, UK
Jp
UK
Ch
Fr
US
Fr
US
Fr
Ir
Ar
Fr
Ch
Pr
US

**Figure 5**

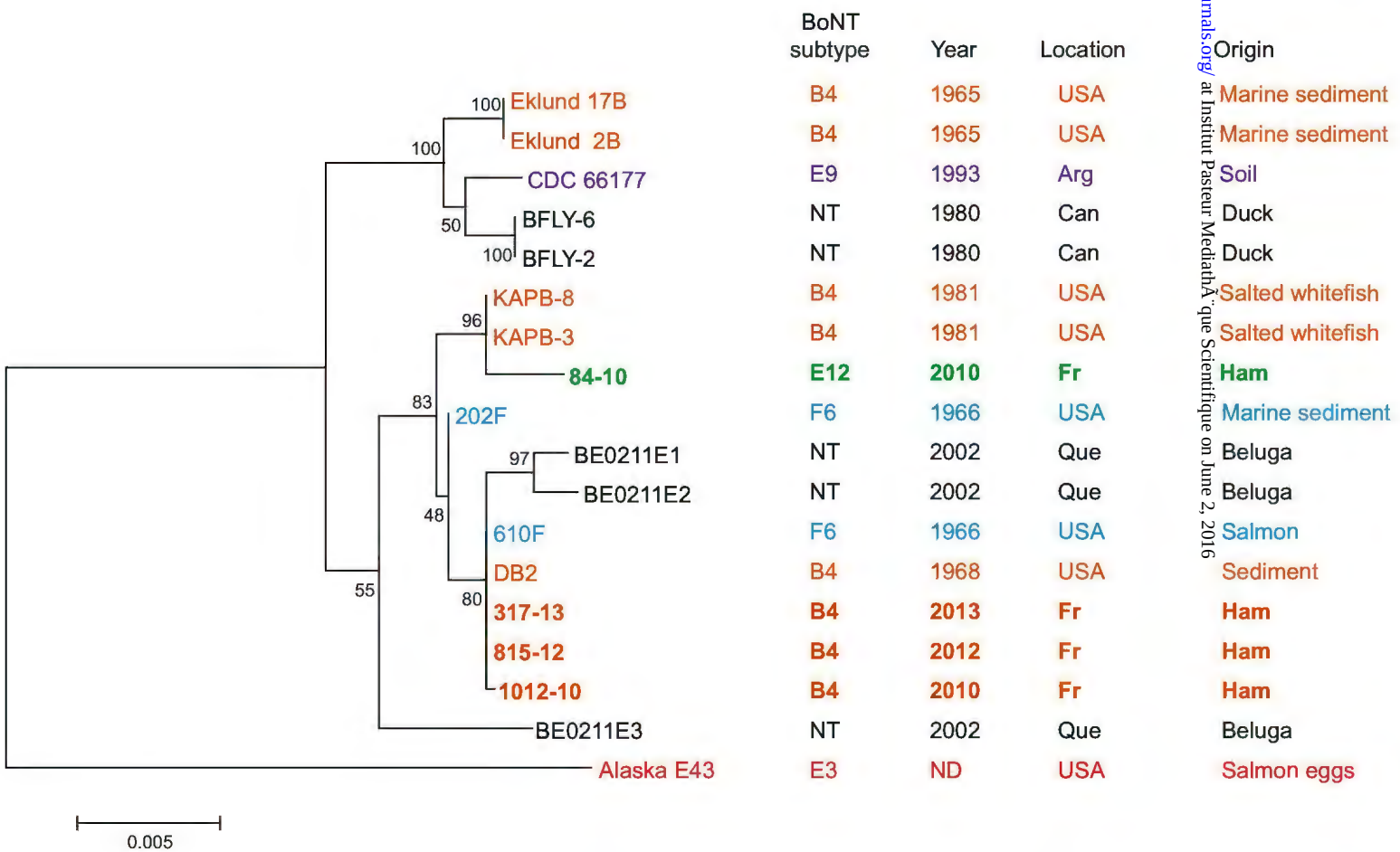


**Figure 6**

e.oxfordjournals.org/ at Institut Pasteur MediathÃ¨que Scientifique on June 2, 2016