

Characterization of the first *Clostridium baratii* strain responsible for an outbreak of botulism type F in France

This article was published in the following Scientific Open Access Journal:

Clinical Microbiology & Case Reports

Received February 23, 2015; Accepted March 06, 2015; Published March 12, 2015

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Abstract

Clostridium baratii strains were isolated from stools of two patients with a severe botulism type F in France. The two strains exhibited the phenotypic characteristics of *C. baratii* and were toxigenic. Whole genome sequencing showed that strain 771-14 contained an *orfX-bontF/7* locus and was highly related to *C. baratii* strain Sullivan.

Botulism is a rare but severe disease mainly resulting from food poisoning or intestinal colonization. The disease is characterized by flaccid paralysis, which can result in severe respiratory distress and death. Botulism is due to potent neurotoxins called botulinum neurotoxins (BoNTs) which are produced by *Clostridium botulinum* and atypical strains of *Clostridium baratii* and *Clostridium butyricum* [1-3]. Based on their immunological properties, BoNTs are classified into 7 toxinotypes (A to G). A new type called H has been reported [4,5] which is rather considered as a mosaic BoNT/FA [6]. However, all these toxins develop similar pathological effects, albeit each toxinotype uses different ways to block the neurotransmitter release. Indeed, BoNTs recognize distinct protein receptors (synaptic vesicle protein SV2 isoforms for BoNT/A, BoNT/E, and BoNT/F, synaptotagmin I and II for BoNT/B and BoNT/G (reviewed in [7]) in addition to gangliosides GD_{1b}/GT_{1b} to enter target neurons, and cleave one of the three proteins (VAMP, SNAP25, syntaxin) of the SNARE complexes which are a key player in the evoked release of neurotransmitter [8-10].

C. baratii producing BoNT/F was first identified as responsible for an infant botulism case in New Mexico (USA) in 1980 [11]. Since this period, several cases of botulism in very young babies due to *C. baratii* type F were reported, mainly in the U.S. [11-13]. *C. baratii* was also recognized as responsible for botulism in adults [13-16]. However, this pathogen remains a rare cause of botulism approximately 1.1% of the botulism cases in the U.S. from 1981 to 2011 [17,18]. Almost all the botulism type F cases linked to *C. baratii* were reported in the U.S., only one outbreak of food-borne botulism in adults due to *C. baratii* was recently reported in Spain (Barcelona) [19].

In France, an outbreak of botulism due to *C. baratii* type F including two women was reported in November 2014 [20]. The two cases presented severe respiratory distress and required respiratory assistance with mechanical ventilation. A high level of BoNT/F (400 mouse lethal doses (MLD)/ml) was identified in serum sample of case 1 (60 years old woman) two days after the onset of symptoms, whereas BoNT/F level in the serum of the second case (20 years old woman) was low (1-2 MLD/ml). Case 1 was completely paralyzed (limbs, ocular and respiratory muscles) and needed respiratory assistance for 46 days, whereas case 2 developed a less severe botulism (diplopia, ptosis, dysphonia, dysphagia, and respiratory distress) and was ventilated during 11 days [20]. BoNT/F was also identified in stool sample from case 1 (160 MLD/g) and was not detected in stool samples from case 2. *C. baratii* was isolated from the stool samples of the two patients using ethanol treatment and Fortified Cooked Meat Medium (FCMM, Difco) agar supplemented with 1% sheep blood incubated at 37°C in anaerobic conditions [21].

The two *C. baratii* strains 771-14 and 777-14 were non-motile Gram-positive rods and produced a lecithinase but not lipase on egg yolk agar. They produced acid from glucose, esculin, fructose, lactose, mannose, maltose, and sucrose. Esculin was hydrolyzed, indole, urease, and gelatinase were not produced. Milk was coagulated and digested. Rapid ID32A galleries (BioMérieux, Marcy-l'Étoile, France) gave the

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profile 0353004010 for the two strains yielding an identification of *C. baratii* at an identification percentage (%ID) of 70% and *Clostridium tertium* at 22%. The two strains were susceptible to penicillin, ampicillin, amoxicillin, ticarcillin, mezlocillin, tetracycline, erythromycin, imipenem, cefalotine, cefotaxime, clindamycin, rifampicin, vancomycin, moxalactam, and moxifloxacin, and resistant to trimethoprim:sulfamethoxazole. BoNT type F was produced in TGY (trypticase, glucose, yeast extract) culture as monitored by mouse bioassay and neutralizing sera against types A, B, C, D, E, and F [21]. Thus, strains 771-14 and 777-14 exhibit the characteristics of the group VI of neurotoxic Clostridia [1].

The draft genome sequence of strain 771-14 was obtained by using a whole genome sequencing (WGS) strategy with an insert length of about 415 bp on average observed on Fragment Analyzer™ Auto CE System (AATI). The WGS library was performed using the Nextera Sample Prep Kit (Illumina, San Diego, CA). The library was then sequenced on MiSeq machine in 158-bases paired-end reads (Illumina, San Diego, CA). Sequence files were generated using Illumina Analysis Pipeline version 1.8 (CASAVA). After quality filtering, 3,183,328 reads were assembled using CLC software version 4 (CLC Bio) yielding 39 contigs > 1000 bp with an average coverage about 200x. N50 was 221,064 bases, N80 73,538 bases and N90 47,330 bases.

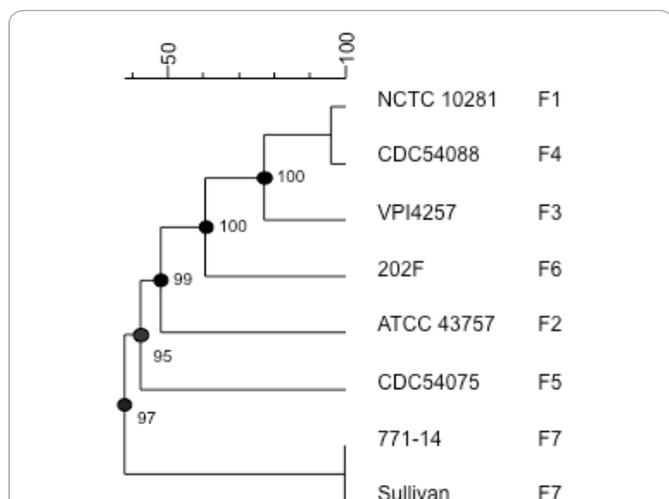


Figure 1. Comparative analysis of BoNT/F from strain 771-14 with representative BoNT/F subtypes at the nucleotide level. Dendrogram was reconstructed from the nucleotide sequence by using the UPGMA method. The genetic distances were computed by using the Kimura two-parameter model. The scale bar indicates similarity values. The number shown next to each node indicates the cophenetic correlation. Evolutionary analyses were conducted in Bionumerics (V.6.6 Applied Maths).

Whole genome sequencing revealed that 16S rRNA gene sequence from strain 771-14 was 99% related to that of *C. baratii*/*C. sardiniense*, and Blast analysis of the contigs showed the presence of only one *bont* locus associated with *orfX* genes. The organization and identity at the nucleotide level of the *bont* locus is similar to that of *C. baratii* strain Sullivan [18]. BoNT produced by *C. baratii* 771-14 and 777-14 is identical at the amino acid level to the other *C. baratii* BoNTs which have been described until now and which are assigned to type F7 [18,22,23]. Phylogenetic relatedness of *bont*/*F7* from strain 771-14 with representative *bont*/*F* subtypes at the nucleotide level is shown in Figure 1. *BotR* gene, which encodes an alternative sigma factor controlling the expression of *bont*, was not found in the *orfX-bont*/*F7* locus of strain 771-14. However, a related gene (88% identity at the amino acid level with BotR from Sullivan strain) was evidenced in a remote position from the botulinum locus on contig 36 (not shown). Similar finding has been reported in *C. baratii* F7 strain IBCA03-0045 as well as in non-proteolytic *C. botulinum* B, E and F6 [22].

Circular representation of chromosomal orthologous genes shows that the strain 771-14 is closely related to strain Sullivan (most of the coding sequences (CDSs) showing $\geq 95\%$ identity) indicating that both strains share related genomic background (Figure 2). In contrast to the strains from the other groups of neurotoxic clostridia [1], the genetic diversity of toxigenic

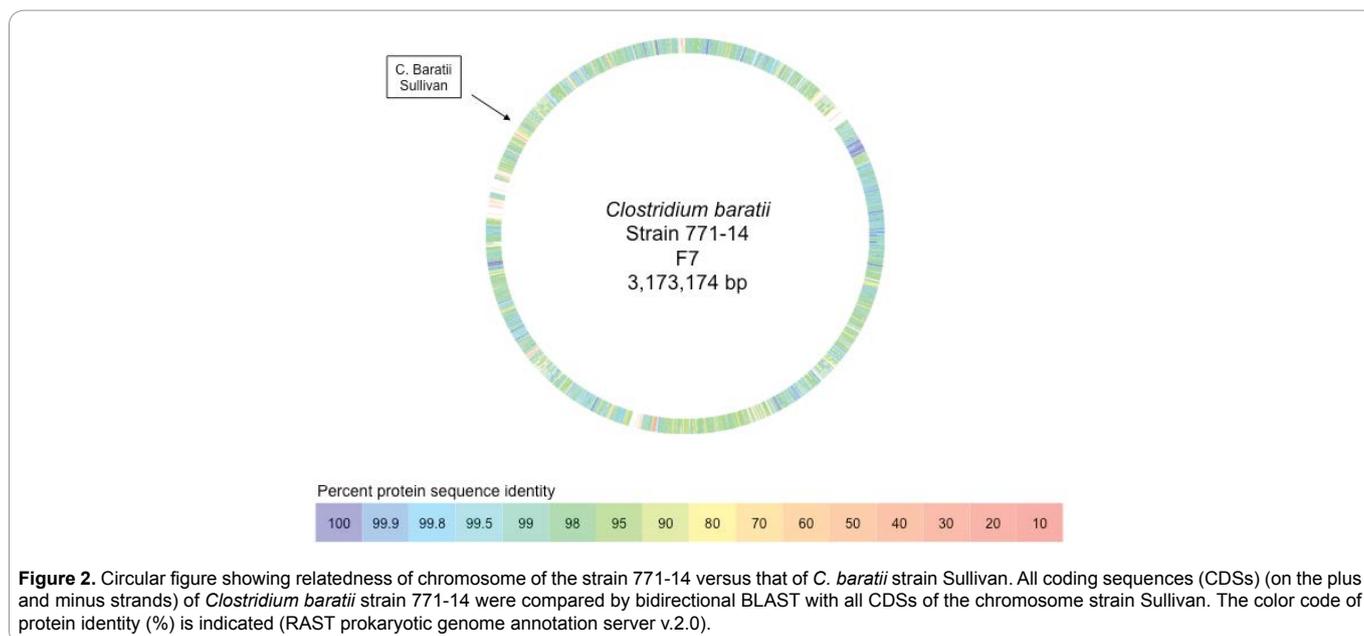


Figure 2. Circular figure showing relatedness of chromosome of the strain 771-14 versus that of *C. baratii* strain Sullivan. All coding sequences (CDSs) (on the plus and minus strands) of *Clostridium baratii* strain 771-14 were compared by bidirectional BLAST with all CDSs of the chromosome strain Sullivan. The color code of protein identity (%) is indicated (RAST prokaryotic genome annotation server v.2.0).

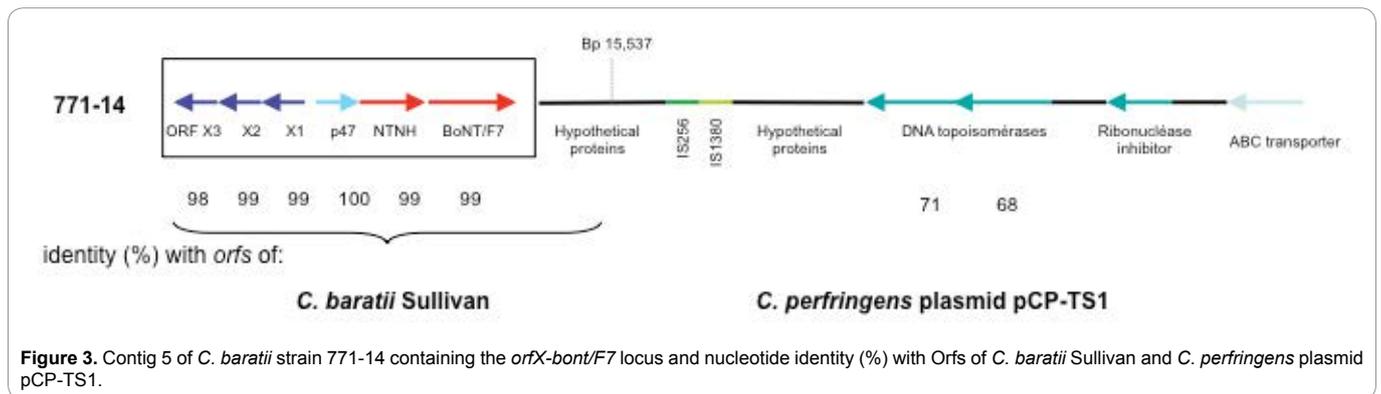


Figure 3. Contig 5 of *C. baratii* strain 771-14 containing the *orfX-bont/F7* locus and nucleotide identity (%) with Orfs of *C. baratii* Sullivan and *C. perfringens* plasmid pCP-TS1.

C. baratii strains seems limited, but only two strains which have a whole genome sequencing available have been compared. Recently, two *C. baratii* strains from two outbreaks of botulism in the New York state were found genetically distinct as monitored by pulsed-field gel electrophoresis and both were different from the reference strain ATCC27638 [15].

A few CDSs of 771-14 display less than 50% identity with those of strain Sullivan, and some gaps are present in strain Sullivan versus strain 771-14 (Figure 1). Interestingly, the *orfX-bont/F7* locus of strain 771-14 lies on a 46,741 bp contig the segment of which (1 to 15,537) shows 99% identity with the corresponding DNA of strain Sullivan (Figure 3). Conversely, the contig part 15,538 to 46,741 bp is unrelated to Sullivan orfs, and 14% of this stretch shares 69% identity with *C. perfringens* plasmid pCP-TS1 including topoisomerase genes and the collagen adhesin (*cna*) gene [23] (Figure 3). Thereby, the *orfX-bont/F7* locus is possibly located on a plasmid in strain 771-14, which remains to be confirmed, but it is not inserted into the same chromosomal site as found in strain Sullivan [18]. Interestingly, a Blast search of IS elements within the contig 5 of the strain 771-14 showed the presence of IS256 and IS1380 downstream the *orfX-bont* locus (Figure 2), instead of IS1182 in strain Sullivan [18,24], supporting a different transfer mechanism of the *orfX-bont/F7* locus into the two strains. Moreover, the insertion site of the *orfX-bont/F7* locus in 771-14 differs from that of *orfX-bont/F6* which lies in *topB* gene in *C. botulinum* [25].

The origin of *C. baratii* botulism is largely unknown. In the France outbreak, all food and drink samples (leftover of pâté, roast beef, mayonnaise, apple tart, and alcopop bottle) were negative for BoNT and neurotoxicogenic *Clostridium* detection [20]. In the literature, the source of contamination was reported in only one outbreak, where toxigenic *C. baratii* was isolated from a tuna can and spaghetti preparation with meat sauce [13]. Botulism type F possibly occurs by intestinal colonization with toxigenic *C. baratii* since most of the patients had a recent gastrointestinal surgery or antibiotic treatment prior to the onset of botulism [17]. However, the unusual high level of toxin in the patient's serum of the France outbreak suggests that she ingested a food containing a high level of toxin. In botulism by intestinal colonization like in infant botulism, BoNT is rarely detected in the serum [26,27], but toxemia in adult with intestinal colonization has not been fully investigated.

The Whole Genome Shotgun project of the strain 771-14 has

been deposited at DDBJ/EMBL/GenBank under the accession number JZTY00000000. The version described in this paper is version JZTY01000000.

Acknowledgements

We thank N. Joly (Projects and Developments in Bioinformatics, Institut Pasteur, Paris) for the reads quality filtering software. High throughput sequencing has been performed on the Genomics Platform, member of "France Génomique" consortium (ANR10-INBS-09-08).

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