Published in final edited form as:

Anaerobe. 2008 December ; 14(6): 318–324. doi:10.1016/j.anaerobe.2008.11.004.

Taxonomy/systematics: *Clostridium taeniosporum* is a close relative of the *Clostridium botulinum* Group II

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Abstract

Clostridium taeniosporum is a Gram-positive, anaerobic, rod-shaped non-toxigenic organism isolated from Crimean lake silt. It is unique in forming spores from which about twelve large, flat, ribbon-like appendages emanate. These ribbon-like structures, about 4.5 μm long and 0.45 μm wide, are assembled from smaller fibrils with 5 nm diameter spherical heads attached to thin tails about 1–2 nm in diameter and about 40 nm in length. The appendages have four major components, a glycoprotein with a collagen-like region, two proteins each of which contains two conserved domains of unknown function, and an ortholog of the *Bacillus subtilis* spore morphogenetic protein SpoVM. Genes for three of these and other, possibly related proteins, cluster on two chromosome fragments. Here we report that *C. taeniosporum* is saccharolytic, non-proteolytic, and produces both acetic and butyric acid fermentation products. It synthesizes α -D-glucosidase and N-acetyl- β -D-glucosaminidase constitutively. These physiological properties are similar to those of the *C. botulinum* Group II. Genotypically, *C. taeniosporum* is also closely related to the same Group II, based on 16S rDNA sequences. *C. taeniosporum* differs from typical *C. botulinum* Group II strains because it is non-toxigenic and in forming the ribbon-like spore appendages. These major differences among otherwise closely related organisms suggest lateral transfer of genes for appendage synthesis and for toxigenicity.

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Keywords

Clostridium taeniosporum; endospore; appendage; 16S rDNA; phylogeny

1. Introduction

Exosporia and appendages are external structures on the endospores of some bacilli and clostridia, as reviewed by Driks [1]. Exosporia are loosely-fitting, sac-like structures which surround the spores, are highly regular in composition, are composed of relatively few proteins (perhaps 10–12), and have no known function [2 – 6]. Appendages were first observed by Krasil'nikov et al. who isolated 19 new species of clostridia and bacilli, all of which formed spores with external appendages [7 – 9]. The appendages have different forms - ribbons, pili, feathers, brushes, tubules and swords - and are attached to spores or to exosporia [7 – 12]. Their functions are also unknown.

One of the new spore-appendage-forming organisms was *Clostridium taeniosporum* [9], isolated from Crimean Lake Mainaki silt. It is especially interesting because about 12 large, ribbon-like appendages (Fig. 1E) are attached through a common trunk at one spore pole to an external coat layer, called the encasement [9, 12, 13]. The ribbons, assembled as a coiled stalk in the mother cell during sporulation [12], are very large, about 4.5 µm long, 0.45 µm wide, and about 30 nm thick [12, 13]. They are thought to be assembled from smaller complexes – fibrils with spherical heads about 5 nm in diameter attached to long, thin 40-nm tails about 1–2 nm in diameter – formed from only four major proteins [13], a 37 kDa glycoprotein, two paralogs of 29 kDa, and an ortholog of the short, 26-residue SpoVM morphogenetic spore component of *Bacillus subtilis* [14].

Here we extend the physiological and phylogenetic characterization of this organism and report that it is a non-toxigenic member of the *Clostridium* cluster Ia [15, 16] and that its nearest neighbors are the non-proteolytic, Group II *C. botulinum* strains [17], based on both phenotypic properties and 16S rDNA similarity.

2. Materials and methods

2.1. Strain and culture conditions

Clostridium taeniosporum strain 1/k was first described as a new species in a paper submitted by Krasil'nikov *et al.*, Soil Biology Faculty, Moscow State University, Moscow, USSR in 1966 but not published until 1968 [9]. The organism was obtained from V. I. Duda, a member of the Krasil'nikov group, by Orville Wyss, Department of Microbiology, University of Texas, Austin, TX, USA, in 1965 and transmitted to L. J. Rode, Jr. of the same department. Rode *et al.* [12] published an electron microscopic study of appendage formation and assembly in 1967, that is, before the Krasil'nikov *et al.* paper [9] appeared in 1968. Spores lyophilized by the Rode group were maintained in the Molecular Genetics & Microbiology Section, University of Texas, and used to culture this organism recently [13]. However, this species was not included in the Approved List and the epithet has not been validly published.

Cells were grown at 30°C under 85% nitrogen, 10% hydrogen, and 5% carbon dioxide in a Forma model 1025 anaerobic chamber, although this organism can withstand about 20 minutes' exposure to air. Spores harvested in an aerobic environment and suspended in water were stored aerobically at 4°C in water for many months without extensive viability loss.

The culture medium was, unless otherwise indicated, modified CDC anaerobe medium [18] containing tryptic soy broth (30 g/l), yeast extract (5 g/l), NaCl (5 g/l), hemin (5 mg/l), vitamin K1 (10 mg/l) and glucose (5 g/l), pH 7.4, with 1.5% agar as needed. Vitamin K1, 10 mg dissolved in 1 ml of absolute ethanol, was added to 1 l of medium after autoclaving. Saccharolytic activity [19] was tested on modified CDC agar plus 1% soluble starch. Plates were incubated for 48 hours and flooded with Gram iodine solution. Colorless zones around the colonies indicated starch hydrolysis. Proteolytic activity was tested on protoplast regeneration medium [20] which was modified CDC agar supplemented with Casamino acids (10 g/l), MgCl₂·6H₂O (5 g/l), CaCl₂·2H₂O (3.7 g/l), gelatin (50 g/l), and bovine serum albumin (8 g/l). Cells were grown in Brain Heart Infusion medium containing 0.1 % sodium thioglycolate [Difco Laboratories, Detroit, MI] for scanning electron microscopy. TPGY medium, used for analysis of botulinum genes by PCR and toxin production by mouse bioassays, contained trypticase peptone (50 g/l), D-glucose (4 g/l), yeast extract (20 g/l) and cysteine. HCl (1 g/l). All media and solutions were reduced in the anaerobic chamber for 24 hr before use.

Constitutive enzyme production was tested with the RapID ANA II system using the manufacturer's protocol (Remel, Lenexa, KS) with cells grown on three different media, modified CDC agar, CDC anaerobe blood agar [18], and Brain Heart Infusion agar, all with the same results. Cells grown for 24 and 48 hrs were scrapped onto a cotton swab, resuspended in RapID Inoculation Fluid, and used to inoculate the test panels. Glycosidase activities were tested using *o*- or *p*-nitrophenyl glycosides. Amino peptidase activities were tested using β -naphthylamide derivatives.

2.2. Staining and microscopy

For fluorescence microscopy, growing cells were stained with both 4,6-diamidino-2-phenylindole (DAPI) [21] and N-(3-triethylammoniumpropyl)-4-(6-(4-(diethylamino)phenyl)hexatrienyl)pyridinium dibromide (FM 4-64) [22] to visualize nucleoids and cytoplasmic membrane, respectively. DAPI and FM 4-64 (Molecular Probes/Invitrogen, Carlsbad, CA) were added at 1 μ g/ml to 1 ml of culture in modified CDC medium at absorbance 0.8 (600 nm). After incubating for 30 minutes in an air atmosphere, cells were concentrated 10-fold in the same solution and visualized using an Olympus BX-60 microscope. The images were captured at 100X with a Photometrics Quantix camera (Roper Scientific, Tucson, AZ) with Metamorph software (Universal Imaging Corp., Downingtown, PA) and processed with Photoshop 7.0 (Adobe Systems Inc. San Jose, CA).

For scanning electron microscopy (SEM) of growing cells, 1 ml of cells at absorbance 0.3 in Brain-Heart Infusion medium containing 0.1 % sodium thioglycolate were fixed in a solution of 2.5 % glutaraldehyde, 2 % formaldehyde and 100 mM sodium cacodylate buffer (pH 7.4) for one hour on ice. The cells were washed and transferred to deionized water. Cells from a 3 μ l-drop were air-dried and coated with a 10 nm-layer of iridium. Images were

captured at 25000X using a Zeiss Supra 40VP field emission scanning electron microscope (Carl Zeiss MicroImaging, Inc., Thornwood, NY) and processed using Photoshop 7.0 (Adobe Systems Inc.). Spores were purified and examined by SEM as described [13]. The image was colored and the background blackened by Photoshop.

2.3. Fermentation product identification and quantitation

Fermentation products were identified and quantitated using a Hewlett Packard 5890 Series II gas chromatograph (Hewlett-Packard Company, Palo Alto, CA), fitted with a Finnigan Mat A200S autosampler system (Thermo Electron Corporation, Waltham, MA), and coupled to a PC installed with Peak 2000 data analysis software (PeakSimple Data Systems, Alltech Associates Inc., Deerfield, IL). Cells were grown in CDC medium modified as above and culture supernatants collected periodically over the absorbance range from 0.2 to 3.0. A sample of CDC medium was included and a solution of standards contained 10mM ethanol, 20 mM acetone, 30mM acetate, 40mM butanol and 30mM butyrate. The supernatants, CDC medium control and the standard solution were acidified by adding 10 μ l of 50% H₂SO₄ per 0.5 ml and 5 μ l samples were injected. Acids and solvents were separated using a temperature gradient program in a 6 ft column (Porapak QS 80/100, 6 ft $\times$ 0.25 in $\times$ 2mm) packed with Deactiglass (part #06087, Alltech Associates Inc., Deerfield, IL). Nitrogen was the carrier. The injector and detector were set to 215°C and 245°C, respectively. The column heating profile was 125°C for 5 minutes, a 30°C/min ramp to 190°C where it was held for 6 minutes, with a final 30°C/min ramp to 220°C, at which it was held for 22 minutes.

2.4. Molecular techniques

Chromosomal DNA was extracted from exponentially growing cells by the Puregene Genomic DNA Purification Kit (Gentra Systems, Minneapolis, MN). A 1.46 kb portion of the 16S rDNA gene was amplified with the Expand High Fidelity Enzyme Mix (Roche Diagnostics, Indianapolis, IN) using generic clostridial rDNA primers [23], purified by a QIAquick PCR Purification Kit [QIAGEN. Inc., Valencia, CA] and sequenced with an Applied Biosystems Model 3730 Automated DNA Sequencer. The sequence has been deposited in GenBank with the accession number **EU696947**.

To test for the presence of botulin neurotoxin and associated genes by PCR, total genomic DNA was isolated from *C. taeniosporum* and *C. botulinum* strains by lysozyme and proteinase K treatment as described previously [24]. DNA was diluted to 50 ng/ μ l for PCR amplifications using the GeneAmp® High Fidelity PCR System (Applied BioSystems). PCR cycles were as follows: 95°C for 2 minutes, followed by 25 cycles of 95°C for 1 minute, an annealing step for 45 seconds at 48°C, 72°C for extension, followed by 1 cycle of 72°C extension for 10 minutes. Extension time depended on the length of the fragment being amplified. Genes tested for, and primers used, [25] are identified in Table 1.

2.5. Phylogenetic analysis

The 1464-nt region of the *C. taeniosporum* 16S rRNA gene, which corresponds to the *E. coli* 16S rDNA positions 30 through 1493 [26], was used as a BLAST version 2.0 [27] query to identify 75 non-redundant similar sequences (Supplementary Table S1; www.sbs.utexas.edu/walker/clostridial_phylogeny) which, along with that of *C. taeniosporum*, were aligned with

CLUSTAL X version 2.0 [28]. Outgroups to root the final trees were the rDNA sequences of *Thermotoga maritima* and *Synechococcus* sp. PCC 6301, chosen because they share a common ancestor with the clostridia and other Gram positive organisms, but separated from that common ancestor earlier than did the clostridia [29]. A distance matrix was generated using the PHYLogeny Interface Package (PHYLP, version 3.67) [30] with the Kimura “2-parameter” model for distance matrix correction [31]. A neighbor-joining tree [32] was then generated with PHYLP; a maximum-likelihood tree [33] was generated with PHYLP DNAML (without assuming a molecular clock). Bootstrap analyses determined the statistical validity of the results and values greater than 70% (based on 1000 replicas) were added to the trees. A Bayesian tree [34] was constructed with Mr. Bayes version 3.1.2 using the Markov chain Monte Carlo algorithm. The general time reversible evolutionary model was selected with gamma-distributed rate variation across sites and a proportion of invariable sites. The analysis was run for 900,000 generations until the standard deviation of split frequencies was less than 0.01. The potential scale reduction factor values were found to be very close to 1.0, indicating a good sample for posterior probability distribution. The values at the nodes indicate the posterior probability of the clades. All trees were manipulated with Dendroscope software, version 1.2.4 [35].

2.6 Toxicity analysis

The mouse bioassay was used to assess the toxicity of *C. taeniosporum*. Cultures grown for four days in TPGY medium were centrifuged to produce culture supernatants or were not centrifuged for use as whole culture samples. Half of the samples were treated with trypsin at 50 ug/ml for 30 minutes at 37°C. The activation process was stopped by adding soybean trypsin inhibitor at the final concentration of 100µg/ml. 0.5 ml of each sample was injected intraperitoneally into each of two mice.

Results and discussion

3.1. Physiological and biochemical properties

C. taeniosporum colonies grew at 30°C on modified CDC medium to 2–3 mm in diameter in 24 hours and to 3–5 mm in 48 hrs. They were white, raised, and moist with irregular edges (Fig. 1A). In liquid medium, the generation time was about 50 min and maximum growth reached an absorbance of 2–3 and a titer about 2×10^8 cells/ml. Exponentially growing cells examined by scanning electron microscopy were long, regular cylinders about 0.8 µm in diameter and 5–10 µm in length. The division plane was perpendicular to the cell long axis, but the division process did not produce cylinders with flat ends. Rather, cells had pointed poles, suggesting that envelope elongation continued during septation (Fig. 1C). Vital staining of growing cells also revealed pointed cylinders 5–10 µm long with a diameter about 1.5 µm, nucleoids covering most of the interior and septa forming near the cell centers (Fig. 1D). A typical spore formed after 8 days' incubation on Brain Heart Infusion agar medium is shown in Fig. 1E. *C. taeniosporum* is saccharolytic (Fig. 1B) and non-proteolytic.

C. taeniosporum produces both acetic and butyric acids. Both were detectable early in exponential growth, when the absorbance was 0.2, and increased in concentration to about 20 and 24 mM, respectively, when the culture reached maximum growth. Constitutive

enzyme production by *C. taeniosporum* is similar to that of the *C. botulinum* Group II members (see below) [36]. It produces α -D-glucosidase and N-acetyl- β -D-glucoseaminidase but does not produce urease, α -L-arabinosidase, β -D-galactosidase, β -D-glucosidase, α -D-galactosidase, α -L-fucosidase, or phosphatase. It does not hydrolyze any amino peptidase tested (leucyl-glycine, glycine, proline, phenylalanine, arginine, serine, or pyrrolidonyl) and does not produce tryptophanase.

3.2. Phylogeny based on the 16S rDNA sequence

C. taeniosporum 16S rDNA was most similar to those of the genus *Clostridium*, corroborating its identification as a *Clostridium* species [9]. These studies also confirmed previous phylogenetic studies of the clostridial Clusters I, II and III [15 – 17]. Seventy Cluster I species or strains, including *Sarcina* and *Anaerobacter* species and unidentified bacteria, were assigned to Subclusters Ia through Ig in neighbor-joining, maximum-likelihood and Bayesian trees. Six Cluster II and III members were also included. The overall topologies of the trees were the same in all three analyses with all organisms positioned within previously described clusters and subclusters, with two exceptions. *C. pascui* was positioned in Subcluster IF in the neighbor-joining and maximum-likelihood trees but in Subcluster IG in the Bayesian tree. The unidentified bacterium clone BSV74 was assigned to Subcluster IA in the neighbor-joining and Bayesian trees but to Subcluster IB in the maximum-likelihood tree.

C. taeniosporum is most closely related to the *C. botulinum* Group II strains (types B, E and F) (17) in all three trees. The 1464 nucleotides of *C. taeniosporum* were 99.1–99.3% identical to those of the neurotoxicogenic, non-proteolytic Group II *C. botulinum* strains [with additional differences of one-nucleotide insertions (at different positions) in *C. botulinum* E ATCC 9564 and *C. botulinum* B Elkund 17B and a two-nucleotide gap in *C. botulinum* E Iwani]. These assignments are strengthened by 100 and 99.9% bootstrap values in the neighbor-joining and maximum-likelihood trees and a posterior probability of 1.0 in the Bayesian analysis. Fig. 2 is a portion of the neighbor-joining tree; complete trees are presented as Supplementary Fig. S1, S2 and S3 (www.sbs.utexas.edu/walker/clostridial_phylogeny).

Although the *C. taeniosporum* 16S rDNA gene is greater than 99% identical to that gene of all four members of the *C. botulinum* Group II, certain 16S rDNA signature nucleotides [37, 38] distinguish *C. taeniosporum* from the Group II *C. botulinum* strains and others distinguish the clade containing *C. taeniosporum* plus the *C. botulinum* Group II from the clade containing *C. kainantoi*, *C. beijerinckii* and closely-related species (Fig. 2). Comparing *C. taeniosporum* to the *C. botulinum* Group II, there are ten positions at which the nucleotide present in *C. taeniosporum* differs from that of the Group II members, all four of which are identical (Fig. 3A). On the other hand, 23 individual or dimeric nucleotides conserved in *C. taeniosporum* plus all four *C. botulinum* Group II members differ from those in the *C. kainantoi* – *C. beijerinckii* clade (Fig. 3B).

Some *C. botulinum* type E strains also form spores with appendages. Hodgkiss & Ordall reported in 1966 [39] that four independently-isolated type E strains all formed spores with numerous, similar, tubular appendages radiating from the spore coat. The tubules were

uniform in diameter (about 20 nm), variable in length (ranging up to about 0.56 μm), and ended in hemispherical caps. The caps were composed of a single layer of 4-nm-diameter spherical subunits attached to the tubule end by an unknown structure. Additionally, the spore coats had flat ridges or bands of rather uniform width raised on the outer surface. An exosporium surrounded both the spores and the appendages. Other relatively close neighbors of *C. taeniosporum* include *C. butyricum* and *C. beijerinckii* (Fig. 2).

It might be argued that a study based on only one gene might not adequately describe the phylogeny of an organism because of the possibilities of lateral gene transfer and mutation [40]. However, the extent of such occurrences is thought to be minimal [41, 42] and phylogenetic studies of the clostridia based on housekeeping gene sequences have corroborated the branching pattern provided by 16S rDNA sequences, reviewed by Strackebrandt and Hippe [16].

3.3. *C. taeniosporum* does not possess any of the known bont genes or produce BoNT

Based on its genealogical similarity to Group II *C. botulinum* strains, *C. taeniosporum* might have been expected to contain wild-type or cryptic botulinum neurotoxin genes or associated cluster genes. Genomic DNA from *C. taeniosporum* and from *C. botulinum* strains known to produce BoNT/A, /B, /E and /F were analyzed for the presence of *bont* and its associated cluster genes by PCR. All the PCR reactions were negative with *C. taeniosporum* DNA, but produced the expected results from the *C. botulinum* control strains. The genes tested and relevant primers [25] are listed in Table 1.

The standard mouse bioassay was used to test for neurotoxin production. *C. taeniosporum* was grown in TPGY medium for four days and used as both the whole culture and as the supernatant. Half of each sample was trypsin activated to ensure that any BoNT present was active and toxic or left untreated. A quantity of 0.5 ml of each sample was injected into each of two mice using intraperitoneal procedures. None of the eight mice demonstrated botulism symptoms, indicating that *C. taeniosporum* does not produce any BoNT proteins.

3.4. Diversity of *C. botulinum*

The *Clostridium* genus is very diverse and includes about twenty individual clusters [15]. About half of the clostridia, including the type species, *C. butyricum*, form cluster I, the cluster to which *C. taeniosporum* and all the *C. botulinum* strains belong. The species *C. botulinum* is itself diverse. For many years, all clostridia which produced botulinum neurotoxin were identified as *C. botulinum*, but those organisms can now be separated into four diverse groups, based on corroborating phenotypic and genotypic analyses. Supplementary Figures S1, S2 and S3 include the four *C. botulinum* Groups [15 – 17, 23]. All members of each of the four *C. botulinum* groups are closely related to each other, but are phylogenetically distant from the other groups [17].

Moreover, all four *C. botulinum* groups contain some genotypically identified strains which are non-toxigenic [17]. For example, some members of *C. botulinum* Group II, based on both phenotype and genotype, do not produce neurotoxin, whereas others do. Finally, some strains of species other than *C. botulinum*, including *C. butyricum* and *C. baratii* are neurotoxic [17]. Collins and East [17] have concluded that neurotoxin production is no

longer useful in *C. botulinum* nomenclature and have proposed that the four *C. botulinum* groups (based on corroborating phenotypic and genotypic analyses) should be designated as separate species. The production of neurotoxin by these organisms or strains of other species could be indicated by a term after the epithet.

3.4. Summary of *Clostridium taeniosporum* characteristics

The epithet “*taeniosporum*” signifies producing spores with large, flat, ribbon-like appendages. It has been pointed out by Dr. Jean P. Euzéby (personal communication) that “*taeniosporum*” is not correct and should be “*taeniosporeum*”. We note, however, that many of the clostridial epithets end in “-sporum” and, according to tradition, we do not propose to change the “*taeniosporum*” epithet.

This Gram-positive, anaerobic organism was isolated from silt of Crimean Lake Mainaki [9]. The cells are uniform rods with pointed ends, about 1.5 µm in diameter (when observed without fixation) and 5–10 µm in length with peritrichous flagella [12]. It forms endospores surrounded by a unique encasement layer [13] which terminates at one pole in a common trunk attached to about 12 large, flat, ribbon-like appendages [9, 12, 13]. *C. taeniosporum* is saccharolytic and non-proteolytic and fermentation products include both acetic and butyric acids. The pattern of constitutive enzyme production and 16S rDNA phylogeny analysis identify *C. taeniosporum* as a close relative of the *C. botulinum* Group II, but it is different from most of the *C. botulinum* strains because it is non-toxigenic and is unique because it forms ribbon-like spore appendages. Some other Group II members form tubular appendages [39]. The facts that both toxin and appendage (ribbons and tubules) production are variable characteristics among a closely related group of clostridia and that neurotoxin genes [17, 43, 44] and spore ribbon-like appendage genes [13] are located in separate clusters suggests that both the toxin and appendage component genes might have been moved by lateral gene transfer, possibly on mobile genetic elements.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Dr. Suzanne S. Barth, Yujia Wen, Hyung Cheol Kim and Vincent Nieto for the RapID ANA II analysis, John Mendenhall and Dwight Romanovicz for scanning electron microscopy, Dr. David Hillis and Derrick Zwicki for help with the phylogenetic tree, Allison Magnum and Cecil Harkey for DNA sequencing, Dr. Jean P. Euzéby for information about the epithet etymology, and Marianna Grenadier for help with the figures. Work in the G. N. B. lab was supported by grants from the National Research Initiative Biobased Products and Bioenergy Production Research of the U. S. D. A. Cooperative State Research, Education and Extension Service (No. 2006-35504-17294) and from the U. S. National Science Foundation (No. BES-0418289). Work in E. A. J. laboratory was sponsored by the NIH/NIAID Regional Center of Excellence for Biodefense and Emerging Infectious Diseases Research (RCE) Programs. The authors acknowledge membership within and support from the Pacific Southwest Regional Center of Excellence grant NIH award 1-U54-AI065359 and the Region V ‘Great Lakes’ RCE (NIH award 1-U54AI057153).

Abbreviation

BoNT botulin neurotoxin

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Fig. 1.

Characteristics of *C. taeniosporum* colonies, cells and an endospore. A. Colonies growing 2 days on modified CDC medium. B. Saccharolytic activity. Colonies growing 2 days on modified CDC medium supplemented with starch were flooded with Gram iodine solution to stain the starch deep blue. The clear areas around the colonies demonstrate starch hydrolysis. C. Scanning electron microscopy of exponentially growing cells collected when the culture absorbance at 600 nm was 0.3. D. Fluorescence microscopy of exponentially growing cells stained with FM 4–64 membrane and DAPI nucleoid stains when the culture absorbance was 0.8. E. SEM of spore with ribbon-like appendages. The image was colored and the background blackened by Photoshop.

Fig. 2.

Neighbor-joining phylogenetic tree of the clostridia closely related to *C. taeniosporum*. The bar represents 0.01 change per nucleotide pair. Bootstrap values are indicated in percentage, based on 1000 repetitions. ^T indicates the type strain. One organism previously identified as *C. acetobutylicum* strain 8052^T has been re-classified as a *C. beijerinckii* strain [45, 46].

Fig. 3.

16S rDNA signature nucleotides and dinucleotide sequences. The horizontal lines represent a portion of the 16S rDNA gene. The nucleotide numbers above the lines correspond to the *C. taeniosporum* sequence; those below the lines are from *E. coli* [26]. A. Comparison of signature nucleotides which differentiate *C. taeniosporum* (above the lines) from the Group II *C. botulinum* strains (letters, below the lines, which are identical in all four strains (Fig. 2)). B. Comparison of signature sequences which differentiate the *C. taeniosporum* – *C. botulinum* Group II clade (letters, above the lines, which are identical in all five members) from the *C. kainantoi* – *C. berjerinckii* clade (letters, below the lines, which were identical in all eight members (Fig. 2), except that the *C. caliptrosporum* sequence over nucleotide number 48 (*E. coli*) is not available, a nucleotide corresponding to *E. coli* number 821 was deleted in *C. caliptrosporum*, and the dinucleotide (*E. coli* positions 1143–1144) is CA in *C. butyricum* only. Interestingly, the nucleotides 615 and 1249 (*E. coli* positions) distinguish the *C. kainantoi* – *C. butyricum* pair from the other six members of the *C. kainantoi* – *C. berjerinckii* clade. Position 615 is G in both *C. kainantoi* and *C. butyricum* and T in all other six members and position 1249 is A in both *C. kainantoi* and *C. butyricum* and C in all other six members.

Table 1

Primers used to identify and characterize the *bont* gene clusters in *C. botulinum* subtypes.

Gene ^a	Primer name ^b	Sequence (5'-3')
ha17	HA17F	ATGTCAGTTGAAAGAACTTTTCTACCT
	HA17R	TTATATTTTTTCAAGTTTGAACATTTGA
ha33	HA33F	ATGGAACACTATTCAGTAATCCAAAATTC
	HA33R	TTATGGGTTACGAATATTCCATTTC
orfx1	ORFX1F	ATGAATCAAACATTTTCTTTTAATTTTGATGACAC
	ORFX1R	TTATGCGATTTGCAATAAATTTTCTTCATTTC
orfx2	ORFX2F	ATGAATAATTTAAACCATTTATATATTACGATTGG
	ORFX2R	TTATTTGCTGTTAGGCTTATTGTTTTTTCTAATG
ntnh	NTNHAF	ATGAATATAAATGACAACCTTAAGTATAAATTC
	NTNHAR	TTATTTAAATGACCATAAATACAAATACTTTGAC
bont/A	ALCF	GAGGTGTAAATATGCCATTGTTAAT
	ALCR	ATCATTAGTAAAATTATCTTCTGAAGC
bont/B	AnyBF	CAGGAGAAAGTGGAGCGAAAAAAG
	AnyBR	TGGTAAGGAATCACTAAAATAAGAAGC
bont/E	E1F	TGTGATCTTAATCATGATATACCCAAA
	E1R	ATTCTCGGAAGCCACAAAAA
bont/F	F1F	GGCATATTTATGGACATTGAAAGA
	F1R	TTGTCCGCGATTGTTTACTG

^aThe HA cluster includes the ha17 and ha33 genes; the Orfx cluster includes the orfx1 and orfx2 genes. Toxin genes for BoNT serotypes /A, /B, /E and /F were analyzed using primers designed to amplify the conserved regions, specifically the light chains, of the various subtypes [25].

^bF, forward; R, reverse