AMERICAN SOCIETY FOR MICROBIOLOGY | mBio®

Check for updates

Botulinum Neurotoxin-Producing Bacteria. Isn't It Time that We Called a Species a Species?

Theresa Smith,[a] Charles H. D. Williamson,[a] Karen Hill,[a,b] Jason Sahl,[a] Paul Keim[a]

[a]The Pathogen and Microbiome Institute, Northern Arizona University, Flagstaff, Arizona, USA
[b]BioScience Division, Los Alamos National Laboratory, Los Alamos, New Mexico, USA

ABSTRACT Botulinum neurotoxins (BoNTs) are produced by a diverse set of seven clostridial species, though alternate naming systems have developed over the last 100 years. Starting in the 1950s, a single-species taxonomy where any bacterium producing BoNT would be designated *Clostridium botulinum* was introduced. As the extreme diversity of these strains was recognized, a secondary system of taxonomic "groups" evolved. It became clear that these groups also had members that did not produce BoNT, and in some cases, they were given formal species names. Genomic analysis now clearly identifies species affiliations whether an isolate is toxigenic or not. It is clear that *C. botulinum* group nomenclature is no longer appropriate and that there are recognized species names for each clostridium. We advocate for the use of the scientific binomials and that the single-species group nomenclature be abandoned.

KEYWORDS *Clostridium botulinum*, botulism, botulinum neurotoxin, phylogenetic analysis, taxonomy

Botulinum neurotoxins are produced by at least seven bacterial groups that meet all the criteria of distinct species (1–5). The history of their discovery has been long and punctuated, which has resulted in convoluted and conflicting taxonomic nomenclature. However, careful examination of the taxonomic precedence reveals that a simple and rigorous naming system is already in place and merely needs to be universally adopted.

THE HISTORY

Emile van Ermengem first described a botulinum neurotoxin (BoNT)-producing organism, which he named *Bacillus botulinus*. He likely believed that botulism was caused by a monospecific toxin produced by a single bacterial strain (6). This was proven erroneous on both counts in 1904 when G. Landmann described another bacterial toxin that had caused botulism following ingestion of contaminated bean salad (7). This was the first recorded case of botulism due to something other than preserved meat products, and it was determined that the toxin and the bacteria producing it differed from the van Ermengem strain (8). The two toxins were serologically distinct, and while van Ermengem's organism was nonproteolytic, the Landmann strain was clearly proteolytic. In 1919, Georgina Burke separated the toxins from several U.S. BoNT-producing strains into two categories, which she designated toxin types A and B (9). The type A toxins appeared to be similar to that of the Landmann toxin, while the type B toxins were similar to the van Ermengem toxin. However, unlike with the van Ermengem strain, all of the U.S. isolates were proteolytic. Thus, it was shown that different bacterial strains may produce the same toxins and different toxins may be produced by the same bacterial strains, a truism that has since been reinforced numerous times.

At about this time, the Committee on Classification of the Society of American

TABLE 1 Phenotypic characteristics of BoNT-producing clostridia

Traditional designation	Produces BoNT	Presence of:					Optimal growth temp (°C)	Proposed designation
		Lipase	Lecithinase	Gelatin liquefaction	Casein digestion	Glucose fermentation		
C. botulinum group I	Yes	+	−	+	+	+	35–40	C. parabotulinum
C. sporogenes	Yes	+	−	+	+	+		C. sporogenes
C. sporogenes	No	+	−	+	+	+		
C. botulinum group II	Yes	+	−	+	−	+	18–25	C. botulinum
C. botulinum group III	Yes	+	−	+	−	+	40	C. novyi sensu lato
C. novyi type A	No	+	+	+	−	+		
C. botulinum group IV	Yes	−	−	+	+	−	37	C. argentinense
C. subterminale	No	−	−	+	+	−		
C. hastiforme	No	−	−	+	+	−		
C. baratii	Yes	−	+	−	−	+	30–45	C. baratii
C. baratii	No	−	+	−	−	+		
C. butyricum	Yes	−	−	−	−	+	30–37	C. butyricum
C. butyricum	No	−	−	−	−	+		

Bacteriologists proposed a genus name change separating the aerobic *Bacillus* species from the anaerobic *Clostridium* species (10), and this new genus designation came into popular usage in the 1920s (11–13).

As more toxin types were being discovered, additional differences among the strains were also noted. In 1922, type C toxin was identified from two different sources. Ida Bengtson isolated a toxin-producing bacterium from the larvae of *Lucilia caesar*, which had been implicated in chicken botulism (14). Similarly, H. R. Seddon reported a novel toxin type associated with cattle forage poisoning (15). While the toxin types were serologically similar, the bacterial strains exhibited different levels of proteolysis, prompting Seddon to designate his proteolytic isolates *Clostridium parabotulinum*, while the somewhat nonproteolytic strains of Bengtson remained *Clostridium botulinum* (15). The terms *C. parabotulinum* and *C. botulinum* continued to be used to differentiate proteolytic from nonproteolytic neurotoxin-producing clostridia for at least the next 30 years, after which A. R. Prevot and E. R. Brygoo (16) proposed designating any botulinum neurotoxin-producing organism *Clostridium botulinum*, based on that single overriding characteristic (J. Gunnison, personal correspondence; see also Fig. S1 in the supplemental material). This single-species designation based on toxin production has been problematic ever since.

THE PROBLEM

Both neurotoxigenic and nonneurotoxigenic members that belong to each of the *C. botulinum* groups, as well as *Clostridium argentinense*, *Clostridium baratii*, and *Clostridium butyricum*, have been identified (2, 5, 17–20). The nontoxigenic isolates that were originally designated *C. botulinum* or *C. parabotulinum* do not fit the strict toxin species designation but nevertheless continued to be designated *C. botulinum*. In addition, there were repeated reports of clostridia that are distinctly different species but that nonetheless possessed the ability to produce botulinum neurotoxins (20–22). Within a few years of the 1953 nomenclature pronouncement, the bacterium that produced type G toxin was found to be sufficiently different from the others to be given its own species name, *C. argentinense* (20, 23). Additional discoveries of a BoNT/F-producing bacterial strain that was clearly the previously described *C. baratii* (21), followed by the isolation of a BoNT/E-producing *C. butyricum* strain (22), violated the convention that all BoNT-producing bacteria should be named *C. botulinum*.

While the single-species designation based upon neurotoxin production simplified BoNT-producing bacterial nomenclature, the strains producing these toxins exhibited a range of differences in their phenotypic characteristics, prompting some to adopt a "group" designation to distinguish among these bacteria for detection and identification purposes (Table 1) (1, 2, 24, 25). Group I included the proteolytic bacteria that had been named *C. parabotulinum*, while group II included nonproteolytic organisms that

produced type B, E, and F toxins. Group III distinguished the type C- and D-producing organisms, and group IV was initially used to describe type G producers. Groups V and VI were briefly given as designators for BoNT-producing *C. baratii* and *C. butyricum* strains, respectively.

GENOME-BASED CLASSIFICATION

Although the single-species designation for *C. botulinum* has remained, there are now opportunities to improve and clarify the variation observed within and among these BoNT-producing bacteria. In particular, innovations in genome sequencing and analysis have revolutionized the way that bacteria can be classified. DNA-DNA hybridization (DDH) techniques were developed in the late 1960s (26), and DDH was accepted as a kind of "gold standard" for taxonomically characterizing bacteria. A review of DDH studies on BoNT-producing clostridia concluded that the genotypic and phenotypic groupings for these bacteria supported each other (2). *C. botulinum* group I members were closely related by DDH methodology, showing the >70% similarity that is considered to be the boundary for a determination to the species level. When tested, some nontoxigenic *Clostridium sporogenes* strains were discovered within this group, while other *C. sporogenes* strains were found to be unrelated to *C. botulinum* group I. Similarly, *C. botulinum* group II bacteria formed a distinct, closely related group. *C. botulinum* group III strains were directly linked to each other, and the pattern held with *Clostridium novyi* and *Clostridium haemolyticum*. *C. botulinum* group IV was designated a distinct species, namely, *C. argentinense*, which is related to some *Clostridium subterminale* strains. In addition, it was discovered that the toxigenic and nontoxigenic strains within both *C. baratii* and *C. butyricum* were otherwise indistinguishable.

Due to technological difficulties surrounding DDH analysis, comparative analysis of 16S rRNA genes, highly conserved genes within all bacteria, succeeded this method (27, 28), and it was widely accepted as the next gold standard in this field. Importantly, analysis of 16S rRNA gene sequences in BoNT-producing clostridia confirmed the previous relationships based upon phenotypic characteristics and DDH techniques (Fig. 1A). (see also Table S1 in the supplemental material) While 16S rRNA gene analysis has proven extremely useful in bacterial evolutionary analysis, it is a single-gene analysis that is less than comprehensive and lacks discriminatory power at lower taxonomic levels.

Whole-genome sequencing (WGS) is now very common and provides the maximum level of genetic resolution for phylogenetic and systematic classification. WGS data analysis methods can target particular genome features and employ different evolutionary models to generate sophisticated insights into bacterial biology. One simple approach that merely uses genome similarity is pairwise average nucleotide identity (ANI) analysis (29). ANI analysis is an *in silico* phenetic methodology that compares bacterial genomic sequences for similarity, in much the same fashion that DDH did in the laboratory. While phenetic methods are considered weaker for phylogenetic inference, they work well for classification when the taxonomic groups are distinct and well separated in evolutionary time. This appears to be true for the BoNT-producing clostridia, and ANI analysis (estimations with Mash [30]) has confirmed earlier species designations and determined that earlier group designations are consistent with distinct species (Fig. 1B).

It is now well documented that there are seven distinct clostridial species capable of producing botulinum neurotoxins and that the botulinum toxin types produced are independent from the bacteria producing them. The idea that these bacterial groups are in fact several distinct species is now widely accepted and has historical precedence (1, 2, 5). The use of arbitrary group names that have no taxonomic status should cease and be replaced by Latin binomial nomenclature that has already been associated with these groups. We suggest the following: (i) that proteolytic *C. botulinum* group I species be referred to as *Clostridium parabotulinum*; (ii) that the *Clostridium botulinum* designation be restricted to the nonproteolytic group II organisms; (iii) that the BoNT/C- and BoNT/D-producing bacteria be included in the newly proposed species "*C. novyi sensu*
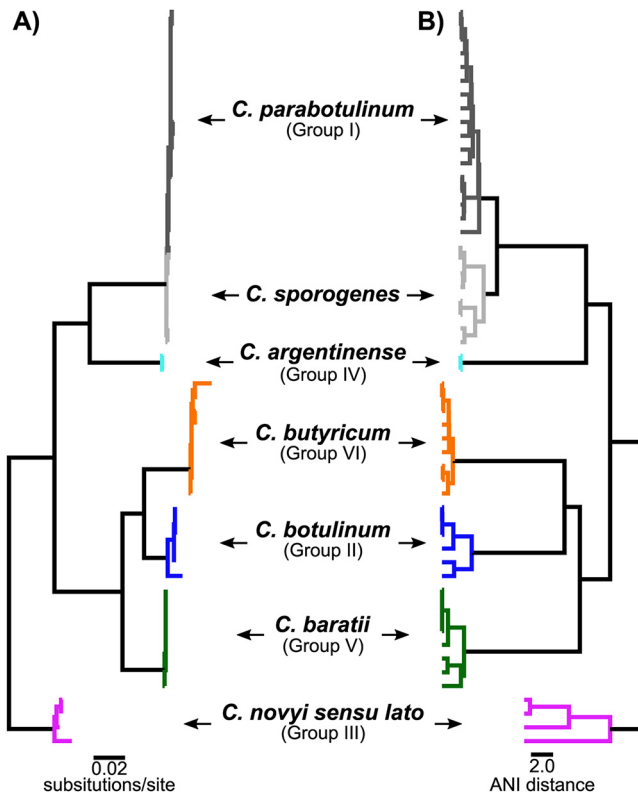
FIG 1 Dendrograms of BoNT-producing bacteria and closely related isolates. DNA sequence analysis of 54 isolates using 16S rRNA gene sequences (aligned with MUSCLE [32]) and maximum-likelihood analysis (IQ-TREE [33, 34]) (A) and using whole-genome sequences to estimate average nucleotide identity (ANI) with Mash ($k = 21$, $s = 1,000,000$) (30) followed by clustering with the unweighted pair group method with arithmetic mean (UPGMA) within QIIME (35) (B). Both methods separate the isolates into 7 taxonomic categories consistent with the historical species designations.

lato" due to their documented close relationship with *C. novyi*, as Skarin et al. proposed (31), which would place these organisms genetically within the larger group of bacteria that includes classic *C. novyi* strains; and (iv) that the remaining BoNT-producing species (*C. argentinense*, *C. baratii*, *C. butyricum*, and *C. sporogenes*) retain their individual species names. As there are both toxic and nontoxic members within each of these species, the proposed changes provide the advantage that they do not rely solely on the expression of botulinum neurotoxin. For clarification, we further propose that BoNT-producing bacterial strains be additionally identified using the toxin type and/or subtype, such as "*C. parabotulinum* BoNT A1" or "*C. baratii* BoNT F," to distinguish between toxic and nontoxic members. Adoption of these proposed species names will assist in clarification of the existing known organisms and provide a framework for the classification of future discoveries.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/mBio .01469-18.

FIG S1, PDF file, 0.1 MB.
TABLE S1, PDF file, 0.05 MB.

REFERENCES

1. Hatheway CL. 1988. Botulism, p 111–133. *In* Balows A, Hausler WH, Ohashi J, Turano A (ed), Laboratory diagnosis of infectious diseases. Springer-Verlag, New York, NY.
2. Collins MD, East AK. 1998. Phylogeny and taxonomy of the food-borne pathogen *Clostridium botulinum* and its neurotoxins. J Appl Microbiol 84:5–17. https://doi.org/10.1046/j.1365-2672.1997.00313.x.
3. Hill KK, Smith TJ, Helma CH, Ticknor LO, Foley BT, Svensson RT, Brown JL, Johnson EA, Smith LA, Okinaka RT, Jackson PJ, Marks JD. 2007. Genetic

diversity among botulinum neurotoxin-producing clostridial strains. J Bacteriol 189:818–832. https://doi.org/10.1128/JB.01180-06.

4. Stackebrandt E, Kramer I, Swiderski J, Hippe H. 1999. Phylogenetic basis for a taxonomic dissection of the genus *Clostridium*. FEMS Immunol Med Microbiol 24:253–258. https://doi.org/10.1111/j.1574 -695X.1999.tb01291.x.

5. Peck MW. 2009. Biology and genomic analysis of *Clostridium botulinum*. Adv Microb Physiol 55:183–265. 320. https://doi.org/10.1016/S0065 -2911(09)05503-9.

6. van Ermengem E. 1897. A new anaerobic bacillus and its relation to botulism. Rev Infect Dis 4:701–719.

7. Landmann G. 1904. Uber die Ursache der darmstadter Bohnenvergiftung. Hyg Rundschau 10:449–452.

8. Leuchs J. 1910. Beitraege zur Kenntnis des Toxins und Antitoxins des *Bacillus botulinus*. Z Hyg Infektionskr 65:55–84. https://doi.org/10.1007/BF02284114.

9. Burke GS. 1919. Notes on *Bacillus botulinus*. J Bacteriol 4:555–571.

10. Winslow C-EA, Broadhurst J, Buchanan RE, Krumwiede C, Rogers LA, Simth GA. 1917. The families and genera of the bacteria—preliminary report of the committee of the Society of American Bacteriologists on characterization and classification of bacterial types. J Bacteriol 2:505–566.

11. Bengtson IA. 1924. Studies on organisms concerned as causative factors in botulism. Hyg Lab Bull 136:1–101.

12. Starin WA, Dack GM. 1925. Pathogenicity of *Clostridium botulinum*. J Infect Dis 36:383–412. https://doi.org/10.1093/infdis/36.4.383.

13. Meyer KF, Gunnison JB. 1929. European strains of *Cl. botulinum* XXXVI. J Infect Dis 45:96–105. https://doi.org/10.1093/infdis/45.2.96.

14. Bengtson I. 1922. Preliminary note on a toxin-producing anaerobe isolated from the larvae of *Lucilia caesar*. Pub Health Rep 37:164–170. https://doi.org/10.2307/4576258.

15. Seddon HR. 1922. Bulbar paralysis in cattle due to the action of a toxicogenic bacillus, with a discussion on the relationship of the condition to forage poisoning (botulism). J Comp Pathol Ther 35:147–190. https://doi.org/10.1016/S0368-1742(22)80021-7.

16. Prevot AR, Brygoo ER. 1953. New investigations on botulinism and its five toxic types. Ann Inst Pasteur 85:544–575. (In French.)

17. Gunnison JB, Meyer KF. 1929. The occurrence of nontoxic strains of *Cl. parabotulinum*, XXXIV. J Infect Dis 45:79–86. https://doi.org/10.1093/infdis/45.2.79.

18. Eklund MW, Poysky FT, Boatman ES. 1969. Bacteriophages of *Clostridium botulinum* types A, B, E, and F and nontoxigenic strains resembling type E. J Virol 3:270–274.

19. Holdeman LV, Brooks JB. 1970. Variation among strains of Clostridium botulinum and related clostridia, p 278–286. *In* Herzberg M (ed), Proceedings of the 1st US-Japan Conference on Toxic Microorganisms. U.S. Government Printing Office, Washington, DC.

20. Suen JC, Hatheway CL, Steigerwalt AG, Brenner DJ. 1988. *Clostridium argentinense* sp. nov.: a genetically homogeneous group composed of all strains of *Clostridium botulinum* toxin type G and some nontoxigenic strains previously identified as *Clostridium subterminale* or *Clostridium hastiforme*. Int J Syst Bacteriol 38:375–381. https://doi.org/10.1099/00207713-38-4-375.

21. Hall JD, McCroskey LM, Pincomb BJ, Hatheway CL. 1985. Isolation of an organism resembling *Clostridium barati* [*sic*] which produces type F botulinal toxin from an infant with botulism. J Clin Microbiol 21:654–655.

22. Aureli P, Fenicia L, Pasolini B, Gianfranceschi M, McCroskey LM, Hatheway CL. 1986. Two cases of type E infant botulism caused by neurotoxigenic *Clostridium butyricum* in Italy. J Infect Dis 154:207–211. https://doi.org/10.1093/infdis/154.2.207.

23. Gimenez DF, Ciccarelli AS. 1970. Another type of *Clostridium botulinum*. Zentralbl Bakteriol Orig 215:221–224.

24. Popoff MR. 1995. Ecology of neurotoxigenic strains of clostridia. *In* Montecucco C (ed), Clostridial neurotoxins, the molecular pathogenesis of tetanus and botulism. Springer-Verlag, Berlin, Germany.

25. Hill KK, Xie G, Foley BT, Smith TJ, Munk AC, Bruce D, Smith LA, Brettin TS, Detter JC. 2009. Recombination and insertion events involving the botulinum neurotoxin complex genes in *Clostridium botulinum* types A, B, E and F and *Clostridium butyricum* type E strains. BMC Biol 7:66–18. https://doi.org/10.1186/1741-7007-7-66.

26. Brenner DJ, Fanning GR, Rake AV, Johnson KE. 1969. Batch procedure for thermal elution of DNA from hydroxyapatite. Anal Biochem 28:447–459. https://doi.org/10.1016/0003-2697(69)90199-7.

27. Stackebrandt E, Ludwig W, Fox GE. 1985. 16S ribosomal RNA oligonucleotide cataloging. Methods Microbiol 18:75–107. https://doi.org/10.1016/S0580-9517(08)70472-0.

28. Woese CR. 1987. Bacterial evolution. Microbiol Rev 51:221–271.

29. Konstantinidis KT, Tiedje JM. 2005. Genomic insights that advance the species definition for prokaryotes. Proc Natl Acad Sci U S A 102:2567–2572. https://doi.org/10.1073/pnas.0409727102.

30. Ondov BD, Treangen TJ, Melsted P, Mallonee AB, Bergman NH, Koren S, Phillippy AM. 2016. Mash: fast genome and metagenome distance estimation using MinHash. Genome Biol 17:132. https://doi.org/10.1186/s13059-016-0997-x.

31. Skarin H, Hafstrom T, Westerberg J, Segerman B. 2011. *Clostridium botulinum* group III: a group with dual identity shaped by plasmids, phages and mobile elements. BMC Genomics 12:1–13. https://doi.org/10.1186/1471-2164-12-185.

32. Edgar RC. 2004. MUSCLE: multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Res 32:1792–1797. https://doi.org/10.1093/nar/gkh340.

33. Nguyen LT, Schmidt HA, von Haeseler A, Minh BQ. 2015. IQ-TREE: a fast and effective stochastic algorithm for estimating maximum-likelihood phylogenies. Mol Biol Evol 32:268–274. https://doi.org/10.1093/molbev/msu300.

34. Kalyaanamoorthy S, Minh BQ, Wong TKF, von Haeseler A, Jermiin LS. 2017. ModelFinder: fast model selection for accurate phylogenetic estimates. Nat Methods 14:587–589. https://doi.org/10.1038/nmeth.4285.

35. Caporaso JG, Kuczynski J, Stombaugh J, Bittinger K, Bushman FD, Costello EK, Fierer N, Peña AG, Goodrich JK, Gordon JI, Huttley GA, Kelley ST, Knights D, Koenig JE, Ley RE, Lozupone CA, McDonald D, Muegge BD, Pirrung M, Reeder J, Sevinsky JR, Turnbaugh PJ, Walters WA, Widmann J, Yatsunenko T, Zaneveld J, Knight R. 2010. QIIME allows analysis of high-throughput community sequencing data. Nat Methods 7:335–336. https://doi.org/10.1038/nmeth.f.303.