HHS Public Access

Author manuscript

Toxicon. Author manuscript; available in PMC 2024 March 01.

Published in final edited form as:

Toxicon. 2023 March 01; 224: 107036. doi:10.1016/j.toxicon.2023.107036.

Unintended consequences: Renaming botulinum neurotoxin-producing species of *clostridium* and related species

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Abstract

Botulinum neurotoxin-producing species of *Clostridium* are highly diverse. *Clostridium botulinum* could represent at least four different species of *Clostridium*. In addition, strains that do not produce botulinum neurotoxin are closely related to toxigenic strains, probably representing the same species. Although reclassification of these organisms has been proposed in the past, their species names have remained unchanged, mainly because of the premise that changing names of medically relevant organisms might cause confusion in the healthcare and scientific community. In this review, we discuss the possible unintended consequences of reclassifying botulinum neurotoxin-producing species of *Clostridium*, which are of public health, medical, and biodefense interest.

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Credit author statement

Carolina Lúquez: Conceptualization, Investigation, Writing – original draft, review, and editing, Jessica L. Halpin: Methodology, Formal analysis, Writing – review & editing, Janet Dykes: Conceptualization, Writing – review & editing.

Ethical statement

Hereby, I Carolina Lúquez consciously assure that for the manuscript "Unintended Consequences: Renaming Botulinum Neurotoxin-Producing Species of *Clostridium* and Related Species", by Carolina Lúquez, Jessica L. Halpin, and Janet Dykes, the following is fulfilled.

1. This material is the authors' own original work, which has not been previously published elsewhere.
2. The paper is not currently being considered for publication elsewhere.
3. The paper reflects the authors' own research and analysis in a truthful and complete manner.
4. The paper properly credits the meaningful contributions of co-authors and co-researchers.
5. The results are appropriately placed in the context of prior and existing research.
6. All sources used are properly disclosed (correct citation). Literally copying of text must be indicated as such by using quotation marks and giving proper reference.
7. All authors have been personally and actively involved in substantial work leading to the paper, and will take public responsibility for its content.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Keywords

Botulinum neurotoxin; *Clostridium botulinum* ; Botulinum neurotoxin-producing clostridia; Taxonomy

1. Introduction

Clostridium botulinum is a Gram-positive, anaerobic, spore-forming bacterium that produces botulinum neurotoxin (BoNT) (Sobel, 2005). BoNT can also be produced by rare strains of *Clostridium baratii* and *Clostridium butyricum* (Sobel, 2005). BoNT and BoNT-producing species of *Clostridium* are of public health and biodefense interest because they present risk of misuse causing mass casualties, thereby posing a severe threat to public health and safety. These organisms are considered Tier 1 Select Agents in the United States, and they are classified as Very High Threat Agents in the European Union (Tian and Zheng, 2014). BoNT blocks the release of acetylcholine at the neuromuscular junction, resulting in paralysis (Sobel, 2005). There are seven serologically distinct types of BoNT: serotypes A through G. Additional serotypes have been proposed (X, En, and H) but have not been accepted by consensus as new BoNT serotypes within the scientific community (Barash and Arnon, 2014; Mansfield et al., 2015; Brunt et al., 2018).

C. botulinum was first described in 1897 by E. van Ermengem, during an investigation of a foodborne botulism outbreak in Belgium. The bacterium was originally named *Bacillus botulinus*, and it was later assigned to the genus *Clostridium* (Collins and East, 1998). Similar organisms isolated during subsequent botulism investigations were later designated as *C. botulinum*. According to the Bergey's Manual of Systematics of Archaea and Bacteria, all those organisms are classified as *C. botulinum* despite their physiological characteristics "because of the unique and similar action of the toxins produced by all strains" (Rainey et al., 2015).

C. botulinum is a highly diverse group of organisms. Historically, the species has been divided into four metabolically distinct groups (Holdeman and Brooks, 1970): Group I, formed by *C. botulinum* type A and proteolytic strains of *C. botulinum* types B and F are proteolytic, grow optimally at 37 °C, and form spores with high heat resistance; Group II, formed by *C. botulinum* type E and non-proteolytic strains of *C. botulinum* types B and F are non-proteolytic, saccharolytic, grow optimally at 30 °C, and form spores with low heat resistance; Group III, formed by *C. botulinum* types C and D are non-proteolytic, grow optimally at 40 °C, and form spores with intermediate heat resistance; Group IV, formed by *C. botulinum* type G are proteolytic, grow optimally at 37 °C, and their spore heat resistance is similar to that of Group III. In addition, other non-BoNT-producing species are closely related to these groups. *C. botulinum* Group I and *C. sporogenes* are closely related; these two species cannot be distinguished by biochemical methods or 16 S rRNA sequences, and can only be identified by toxin neutralization tests in mice (Rainey et al., 2015). Group III strains and *Clostridium novyi* type A are closely related; moreover, *C. botulinum* type C can be cured of type C phage and converted to *C. novyi* type A following infection by a *C. novyi* type A phage (Rainey et al., 2015). Also, Group IV strains are closely related to

Clostridium subterminale. Suen et al. (1988) proposed renaming *C. botulinum* Group IV as *C. argentinense*, a new species which would include nontoxigenic strains of *Clostridium subterminale* and *Clostridium hastifome*.

Reclassification of BoNT-producing species of *Clostridium* has been discussed in the past, as each of the four Groups could represent separate species (Collins and East, 1998), but the name has remained unchanged, mainly because of the premise that changing names of medically relevant organisms can cause confusion in the healthcare and scientific community (Lawson et al., 2016). In 1998, Collins and East (1998) proposed that, because of the clinical and veterinary importance of *Clostridium botulinum*, any future nomenclature must consider BoNT production, in addition to phenotypic and genotypic information. Recently, in 2018, Smith et al. (Smith et al., 2018) proposed that seven distinct species of *Clostridium* would be capable of producing BoNT. The authors proposed that the current metabolic group designations should be replaced by the following classification: 1) *C. botulinum* Group I and related strains of *C. sporogenes* should be referred to as *C. parabotulinum*; 2) *C. botulinum* Group II should be referred to as *C. botulinum*; 3) *C. botulinum* Group III and related strains of *novyi* type A should be referred to as *C. novyi sensu lato*; and 4) *C. argentinense*, *C. baratii*, *C. butyricum*, and *C. sporogenes* should retain their current species names (Smith et al., 2018). This new classification has not been formally accepted, as it has not been recorded on the validation lists published by the International Journal of Systematic and Evolutionary Microbiology (https://www.bacterio.net/ and https://lpsn.dsmz.de/). In this review, we discuss possible unintended consequences of reclassifying these organisms.

1.1. C. botulinum *Group I and related species*

Recent studies using whole genome sequence analyses have confirmed a marked similarity between *C. botulinum* and *C. sporogenes*. For instance, Weigand et al. in 2015 (Weigand et al., 2015) analyzed the genomes of 28 strains of *C. sporogenes* and 9 strains of *C. botulinum* Group I by core genome phylogeny and variable gene content analysis. The genomes formed two separate clades; 5 of the 9 *C. botulinum* strains were more closely related to *C. sporogenes* strains than to other *C. botulinum* Group I strains, and 2 of the 28 *C. sporogenes* strains were more closely related to *C. botulinum* Group I than to other *C. sporogenes* strains. The Average Nucleotide Identity (ANI) between the two clades was 93%, which is below 95%, the cutoff value frequently used for species demarcation (Kim et al., 2014). Weigand et al. (2015) also suggested that *C. sporogenes* clade-specific genes could provide a genomic signature for “true” *C. sporogenes* strains. This approach could be a useful research tool but would require sequencing isolates before classifying them as *C. sporogenes* or *C. botulinum*, which could delay botulism case investigations.

Similarly, Williamson et al. (2016) reported in 2016 that four *C. sporogenes* and five *C. botulinum* type B strains clustered together by core genome phylogeny. The study also reports two clades within Group I. ANI values were above 95% within each of the two clades, and fell below 95% (minimum, ~92%) when comparing all strains within Group I. The authors proposed that the ability to produce BoNT type B within the *C. sporogenes*/*C.*

botulinum type B clade appears to be plasmid-mediated. A recent study by Wentz et al., published in 2021 provides data supporting this idea (Wentz et al., 2021).

A study by Cruz-Morales et al. (2019) reported in 2019, analyzed 779 genomes of *Clostridium*, including 106 genomes of *Clostridium botulinum* Group I (types A, B, and F) and *C. sporogenes*, by core genome phylogeny, using 27 conserved proteins among those genomes. Based on those 27 conserved proteins, the study showed that strains *C. botulinum* types A, B, and F and *C. sporogenes* clustered together, separated from other *C. botulinum* strains. Further analysis revealed that this subgroup had an open pangenome, meaning that the number of new gene families continuously increased in this lineage, demonstrating larger genetic diversity. The observations from this study support the presence of distinct lineages among *C. botulinum* strains.

In a more recent study published in 2020, Brunt et al. (2020a) reported a comparative genomic analysis of 556 strains of *C. botulinum* Group I and *C. sporogenes*, using core genome single-nucleotide polymorphism. The study reported that 23 of 452 (5%) strains assigned to a *C. botulinum* Group I lineage did not possess a *bont* gene, and that the genome of 20 of 104 (19%) of strains assigned to a *C. sporogenes* lineage possessed *bont* gene(s). Brunt et al. also reported observing two main clades within Group I. The study did not report ANI values.

1.2. C. botulinum *Group II and related species*

Williamson et al. (2016) reported in 2016 the analysis of 15 genomes of *C. botulinum* Group II by core genome single-nucleotide polymorphism analysis. They found that the 15 genomes were divided into two distinct clades; one clade contained *C. botulinum* type E only, and the other clade included *C. botulinum* types B, E and F. ANI values within each clade were greater than 97%, and ANI for all strains fell below the species-delineating threshold of 95% (minimum of ~94%). The study did not include non-toxigenic strains.

Cruz-Morales et al. (2019) reported in 2019 the analysis of 779 genomes of *Clostridium*, including 20 genomes of *C. botulinum* type E by core genome phylogeny, and showed that this group clustered separately from other *C. botulinum* strains. The study also indicates that these strains had an almost closed pangenome, implying loss of genetic diversity, as fewer gene families were being added to the pangenome.

Brunt et al. (2020b), reported in 2020 the analysis of 208 genomes of non-proteolytic *C. botulinum* strains by core genome single-nucleotide polymorphism. They also reported two major lineages, one with type E-producing strains only, the other one with strains producing BoNT types B, E or F. ANI was not calculated. In addition, the study reported that 31 of the 208 (15%) strains did not harbor a *bont* gene but still clustered with *C. botulinum* Group II strains. Both major lineages included non-toxigenic strains. The strains lacking a *bont* gene were closely related to strains possessing the *bont* gene on a plasmid or the chromosome. Although no specific name has been used to describe these strains, non-toxigenic organisms that resemble *C. botulinum* Group II have been reported (Collins and East, 1998). Unfortunately, most of the non-toxigenic strains used in the study by Brunt

et al. did not list a known source, country, or year of isolation; therefore, epidemiological inferences of the observed similarity are limited.

1.3. C. botulinum *Group III and related species*

Skarin et al. (2011) reported in 2011 the analysis of whole genome sequences of six strains of *C. botulinum* Group III and one strain of *C. novyi* A by average similarity of the conserved core. The study reports that the genome of the *C. novyi* strain belonged to the same lineage as *C. botulinum* type C strains. The authors proposed classifying *C. botulinum* as dual species: a pathospecies *C. botulinum*, which would include all BoNT-producing strains (types A through G), and a genospecies *C. novyi sensu lato* which would include *C. botulinum* Group III, *C. novyi* and *C. haemolyticum* (Skarin et al., 2011). This proposed classification has not been formally recorded on the lists published by the International Journal of Systematic and Evolutionary Microbiology (https://www.bacterio.net/ and https://lpsn.dsmz.de/).

Skarin and Segerman (2014) also showed that 24 strains of *C. botulinum* Group III, *C. novyi*, and *C. haemolyticum* were closely related when analyzed by pairwise average BLASTN score similarities. Genomic comparisons of the 24 genomes and 61 plasmids revealed four separate lineages, which did not strictly correlate with the species designations, highlighting the genomic complexity within the *C. novyi sensu lato* group (Skarin and Segerman, 2014). Lineage I included only *C. botulinum* group III strains, while lineages II, III, and IV included strains of *C. botulinum*, *C. haemolyticum*, and *C. novyi*.

In 2019, Cruz-Morales et al. (2019) reported the analysis of 779 genomes of *Clostridium*, including 42 genomes of *C. botulinum* types C, D, and mosaic CD, *C. haemolyticum*, and *C. novyi*, and showed high synteny among these strains. In addition, the study showed that this group has an almost closed pangenome, meaning that a few gene families were added to the pangenome, suggesting loss of genetic diversity.

In a more recent study reported in 2021, Fillo et al. (2021) used overall genomic similarity to analyze the phylogenetic relations among genomes of 60 *C. botulinum* types C, D, C/D, and D/C (newly sequenced) and the genomes of 36 *C. botulinum*, 9 *C. novyi*, and 3 *C. haemolyticum* (previously sequenced). The same four lineages mentioned above were also identified by Fillo et al., who further divided lineage I into two branches, IA and IB. Analysis of the botulinum neurotoxin gene revealed that the four BoNT serotypes produced by *C. botulinum* group III (C, D, C/D and D/C) were highly conserved; no new subtypes were identified.

1.4. C. botulinum *Group IV and related species*

Worldwide, few BoNT type G-producing strains have been identified; these strains have been classified as *C. botulinum* type G or *C. argentinense*. Suen et al. (1988) reported in 1988 the use of DNA hybridization studies to characterize 9 strains of *C. botulinum* type G, 11 strains of *C. subterminale*, 3 strains of *C. hastiforme*, and several other strains of *C. botulinum* types A, B, and F, *C. sporogenes*, and other related species. The study showed that all nine *C. botulinum* type G strains, two *C. subterminale* strains, and one *C. hastiforme* strain were included in the same hybridization group, sharing 94% intragroup

relatedness. The authors proposed renaming *C. botulinum* Group IV as *C. argentinense*, a new species that would include non-toxigenic strains of *C. subterminale* and *C. hastiforme* (Suen et al., 1988). *C. argentinense* has since been formally recorded on the lists published by the International Journal of Systematic and Evolutionary Microbiology (https://www.bacterio.net/ and https://lpsn.dsmz.de/). No phylogenetic analyses of *C. argentinense* have been published recently.

2. Relatedness among *C. botulinum* and related species by ANI analysis

ANI is used to calculate the relatedness between genome sequences, to determine if they belong to the same or separate species. The proposed and generally accepted species boundary for ANI values is ~95% (Chun et al., 2018). We used Mashtree v.0.37 (Katz et al., 2019) to find nearest neighbors among representatives of metabolic groups I, II, and III (Fig. 1); we also determined ANI for eight clusters within metabolic groups I, II, and III (Table 1) by using publicly available sequences. Unfortunately, whole genome sequences from only two of the *C. argentinense* strains characterized by Suen et al. (Holdeman and Brooks, 1970) were publicly available; thus, ANI was not calculated for this group. In addition, ANI was not calculated for *C. butyricum* type E and *C. baratii* type F as no substantial genomic variability has been shown between toxigenic and non-toxigenic strains within the two species, and the two groups are clearly separated from other BoNT-producing species of *Clostridium* (Smith et al., 2018).

Clusters 1 and 2 within metabolic group I, which include *C. botulinum* type A, proteolytic *C. botulinum* types B and F, and *C. sporogenes* strains, yielded an average ANI value of 93.1% between the two clusters (maximum 93.9%) (Fig. 2), confirming findings from other authors that the ANI between these two clusters fall outside values corresponding to a single species (Weigand et al., 2015; Williamson et al., 2016).

Clusters 3 and 4 within metabolic group II, which include *C. botulinum* type E, non-proteolytic *C. botulinum* types B and F, and non-toxigenic strains, resulted in an average ANI value of 94% (maximum 94.1%) (Fig. 3), slightly below the species threshold of 95%.

Clusters 5, 6, 7, and 8 within metabolic group III, which include *C. botulinum* types C, D, C/D, and D/C, *C. novyi*, and *C. haemolyticum*, resulted in the following average ANI values (Fig. 4): 92.3% (maximum 92.8%) between clusters 5 and 6; 86.1% (maximum 87.8%) between clusters 5 and 7; 85.3% (maximum 85.8%) between clusters 5 and 8; 85.9% (maximum 86.9%) between clusters 6 and 7; 85.5% (maximum 85.9%) between clusters 6 and 8; and 90.7% (maximum 91%). These results do not support the proposal of Skarin et al. (2011) to include *C. botulinum* Group III, *C. novyi* and *C. haemolyticum* as part of the genospecies *C. novyi sensu lato*. As ANI values among these clusters fall below the species threshold of 95%. This discrepancy might be explained by the different methods employed: ANI in this report, and conserved core genome by Skarin et al. (2011).

3. Reclassification of BoNT-producing species of *clostridium*

Reclassification of *Clostridium botulinum* and related species has been proposed several times, to separate *C. botulinum* strains as distinct species and to include non-toxigenic

strains as part of those new species. For instance, Bengtson in 1924 (Bengston, 1924) was the first to propose separating proteolytic and non-proteolytic strains of *C. botulinum* as *C. parabotulinum* and *C. botulinum*, respectively. However, Prévot (Pa, 1953) published in 1953 an opinion letter suggesting that all organisms that produce BoNT should be classified as *C. botulinum*. In 1988, Suen (Suen et al., 1988) proposed that *C. botulinum* type G should be designated as *C. argentinense*, which also includes a few strains of *C. subterminale* and *C. hastifome*. In 1998 Collins and East (1998) proposed that *C. botulinum* Groups I–IV should constitute separate species; the authors also proposed adding the term "variety" followed by the toxin type to designate toxigenic strains. In 2011, Skarin et al. (2011) proposed classifying *C. botulinum* Group III as dual species: a pathos-pecies, *C. botulinum*, which would include all BoNT-producing strains (types A through G), and a genospecies, *C. novyi sensu lato*, to include *C. botulinum* Group III, *C. novyi*, and *C. haemolyticum*. More recently, in 2018, Smith et al. (2018) proposed a reclassification that would divide *C. botulinum* into four separate species, and which would add *C. sporogenes* to the list of BoNT-producing species of *Clostridium*.

Despite the evidence supporting a reclassification of BoNT-producing species of *Clostridium*, no formal decision has been made and the scientific community has not yet reached consensus regarding the taxonomic classification of this highly diverse group of organisms. According to Roney et al. (Rainey et al., 2015) "*In any other group of organisms, this species would have been divided into four separate species because of the distinct differences in metabolic activity exhibited by strains in the four groups and the lack of DNA homology among groups. However, because of the unique and similar action of the toxins produced by all strains and to facilitate communication between the microbiological and medical professions, they have been retained in one species*". As shown in Table 1, BoNT-producing species of *Clostridium* could be divided into eleven distinct phylogenetic clades. It would be impractical to give each of those clades a separate species name because all produce BoNT; therefore, they can cause botulism. On the other hand, three of those clades already have a unique species name: *C. argentinense*, *C. baratii*, and *C. butyricum*. The metabolic and microbiological properties of these organisms differ greatly from *C. botulinum*. Moreover, *C. butyricum* and *C. baratii* were recognized as separate species well before the first strains able to produce BoNT were discovered, thus they retained their original species names although they belong to the group of BoNT-producing species of *Clostridium*.

Another potential consequence of reclassifying BoNT-producing species of *Clostridium* would be to substantially delay laboratory test results if isolates would have to be sequenced before they could be reported as belonging to a particular species. Moreover, public health laboratories may not have the capacity to characterize BoNT-producing species of *Clostridium* by whole genome sequencing, further delaying the reporting of results. This is of particular importance in those instances when laboratory confirmation of botulism is achieved by isolation of *C. botulinum*; e.g., serum specimen is not available for toxin detection testing or it is negative for BoNT, and *C. botulinum* is isolated from a stool specimen. Knowing the isolate's specific group (or species) might be valuable for research purposes, but this information would not contribute to clinical management as treatment of botulism does not differ by strain.

BoNT-producing species of *Clostridium* are listed as Tier 1 Select Agents in the United States. According to current regulations, BoNT-producing species of *Clostridium* include the following species: *C. botulinum*, *C. baratii*, *C. butyricum*, and *C. argentinense*. Historically, *C. botulinum* includes all organisms that produce BoNT and present relevant microbiological characteristics (anaerobic, lipase positive, etc.), and *C. sporogenes* includes those organisms that microbiologically resemble *C. botulinum* but do not produce BoNT. Thus, reclassifying *C. sporogenes* and *C. botulinum* to include both toxigenic and non-toxigenic strains would require updating the current Select Agents and Toxins regulations. Such changes might not be justified as rule 56a (Brunt et al., 2018) of the International Code of Nomenclature of Prokaryotes (Parker et al., 2019) states that "*names whose application are likely to lead to accidents endangering health or life or both or of serious economic consequences*" can be rejected.

Moreover, including toxigenic and non-toxigenic in the same species, as the classification noted above proposes, could have adverse consequences. For instance, if a non-toxigenic organism identified as "*C. botulinum"* by whole genome sequencing was isolated from a commercially-produced food item such as infant formula, it could be misinterpreted as an actionable finding, *i.e.*, safety recall of the product.

4. Conclusions

Since a reclassification of these organisms will not improve clinical or public health measures, we propose that the species name of *C. botulinum* remain unchanged, to minimize the risk of miscommunication among the public health, medical, and scientific communities. Alternatively, the term "genospecies" could be used when using classifications other than the currently accepted. For instance, "*C. botulinum* type B, genospecies *C. sporogenes*" would indicate that the strain produces BoNT type B and belongs to clade 2, with other non-toxigenic *C. sporogenes* strains.

Acknowledgements

This project was supported by the Center for Preparedness Response, Centers for Disease Control and Prevention. The findings and conclusions in this report are those of the authors and do not necessarily represent the views of the Centers for Disease Control and Prevention.

Data availability

Data will be made available on request.

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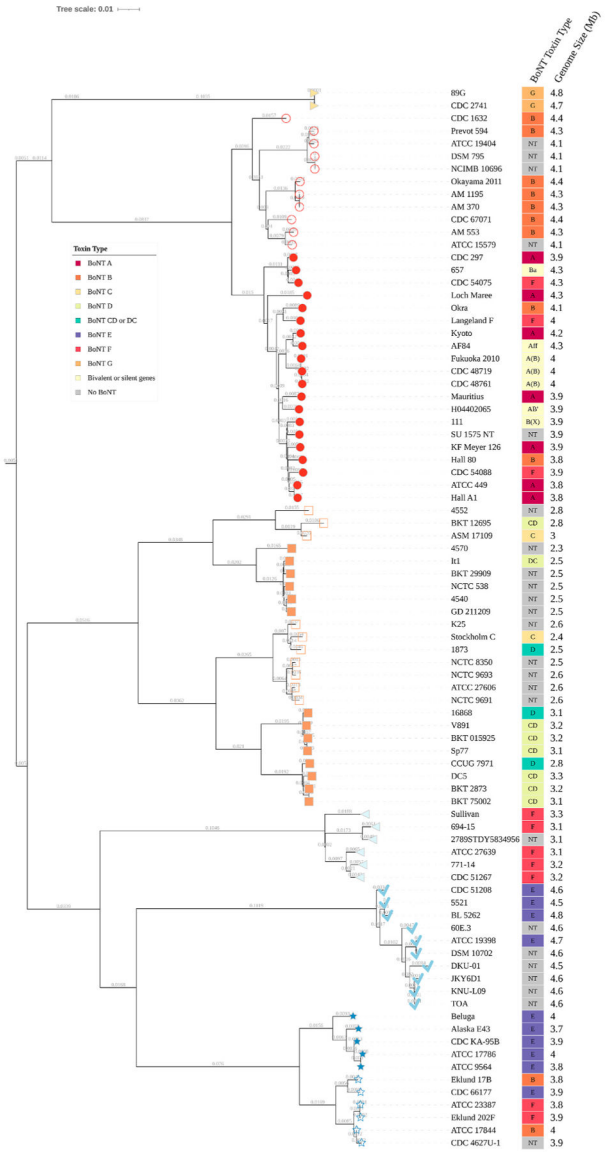
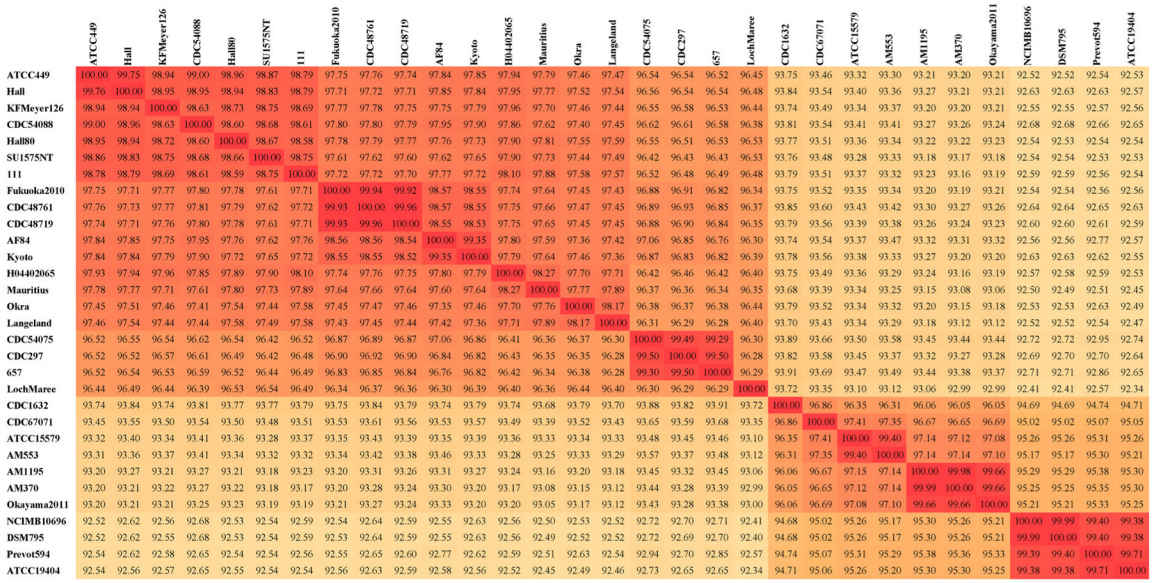


Fig. 1.

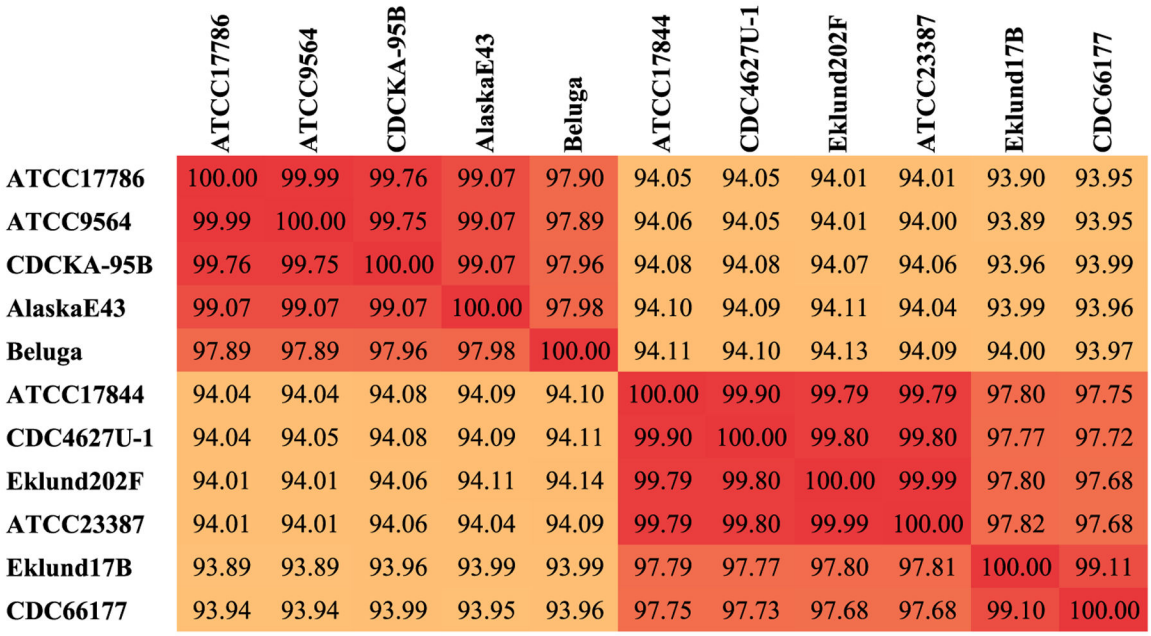
Neighbor-Joining tree drawn using mash distances between whole genome sequences. Tree created using mashtree 0.37 and annotated with the Interactive Tree of Life v. 6.5.4 (Letunic and Bork, 2021).



	ATCC449	Hall	KFMeyer126	CDC54088	Hall80	SU1575NT	111	Fukuoka2010	CDC48761	CDC48719	AF84	Kyoto	H04402065	Mauritius	Okra	Langeland	CDC54075	CDC297	657	LochMaree	CDC1632	CDC67071	ATCC15579	AM553	AM1195	AM370	Okayama2011	NCIMB10696	DSM795	Prevot594	ATCC19404
ATCC449	100.00	99.75	98.94	99.00	98.96	98.87	98.79	97.75	97.76	97.74	97.84	97.85	97.94	97.79	97.46	97.47	96.54	96.54	96.52	96.45	93.75	93.46	93.32	93.30	93.21	93.20	93.21	92.52	92.52	92.54	92.53
Hall	99.76	100.00	98.95	98.95	98.94	98.83	98.79	97.71	97.72	97.71	97.85	97.84	97.95	97.77	97.52	97.54	96.56	96.54	96.54	96.48	93.84	93.54	93.40	93.36	93.27	93.21	93.21	92.63	92.63	92.63	92.57
KFMeyer126	98.94	98.94	100.00	98.63	98.73	98.75	98.69	97.77	97.78	97.75	97.75	97.79	97.96	97.70	97.46	97.44	96.55	96.58	96.53	96.44	93.74	93.49	93.34	93.37	93.20	93.20	93.21	92.55	92.55	92.57	92.56
CDC54088	99.00	98.96	98.63	100.00	98.60	98.68	98.61	97.80	97.80	97.79	97.95	97.90	97.86	97.62	97.40	97.45	96.62	96.61	96.58	96.38	93.81	93.54	93.41	93.41	93.27	93.26	93.24	92.68	92.68	92.66	92.65
Hall80	98.95	98.94	98.72	98.60	100.00	98.67	98.58	97.78	97.79	97.77	97.76	97.73	97.90	97.81	97.55	97.59	96.55	96.51	96.53	96.53	93.77	93.51	93.36	93.34	93.22	93.22	93.23	92.54	92.53	92.54	92.54
SU1575NT	98.86	98.83	98.75	98.68	98.66	100.00	98.75	97.61	97.62	97.60	97.62	97.65	97.90	97.73	97.44	97.49	96.42	96.43	96.43	96.53	93.76	93.48	93.28	93.33	93.18	93.17	93.18	92.54	92.54	92.53	92.53
111	98.78	98.79	98.69	98.61	98.59	98.75	100.00	97.72	97.72	97.70	97.77	97.72	98.10	97.88	97.58	97.57	96.52	96.48	96.49	96.48	93.79	93.51	93.37	93.32	93.23	93.16	93.19	92.59	92.59	92.56	92.54
Fukuoka2010	97.75	97.71	97.77	97.80	97.78	97.61	97.71	100.00	99.94	99.92	98.57	98.55	97.74	97.64	97.45	97.43	96.88	96.91	96.82	96.34	93.75	93.52	93.35	93.34	93.20	93.19	93.21	92.54	92.54	92.56	92.56
CDC48761	97.76	97.73	97.77	97.81	97.79	97.62	97.72	99.93	100.00	99.96	98.57	98.55	97.75	97.66	97.47	97.45	96.89	96.93	96.85	96.37	93.85	93.60	93.43	93.42	93.30	93.27	93.26	92.64	92.64	92.65	92.63
CDC48719	97.74	97.71	97.76	97.80	97.78	97.61	97.71	99.93	99.96	100.00	98.55	98.53	97.75	97.65	97.45	97.45	96.88	96.90	96.84	96.35	93.79	93.56	93.39	93.38	93.26	93.24	93.23	92.60	92.60	92.61	92.59
AF84	97.84	97.85	97.75	97.95	97.76	97.62	97.76	98.56	98.56	98.54	100.00	99.35	97.80	97.59	97.36	97.42	97.06	96.85	96.76	96.30	93.74	93.54	93.37	93.47	93.32	93.31	93.32	92.56	92.56	92.77	92.57
Kyoto	97.84	97.84	97.79	97.90	97.72	97.65	97.72	98.55	98.55	98.52	99.35	100.00	97.79	97.64	97.46	97.36	96.87	96.83	96.82	96.39	93.78	93.56	93.38	93.33	93.27	93.20	93.20	92.63	92.63	92.62	92.55
H04402065	97.93	97.94	97.96	97.85	97.89	97.90	98.10	97.74	97.76	97.75	97.80	97.79	100.00	98.27	97.70	97.71	96.42	96.46	96.42	96.40	93.75	93.49	93.36	93.29	93.24	93.16	93.19	92.57	92.58	92.59	92.53
Mauritius	97.78	97.77	97.71	97.61	97.80	97.73	97.89	97.64	97.66	97.64	97.60	97.64	98.27	100.00	97.77	97.89	96.37	96.36	96.34	96.35	93.68	93.39	93.34	93.25	93.15	93.08	93.06	92.50	92.49	92.51	92.45
Okra	97.45	97.51	97.46	97.41	97.54	97.44	97.58	97.45	97.47	97.46	97.35	97.46	97.70	97.76	100.00	98.17	96.38	96.37	96.38	96.44	93.79	93.52	93.34	93.32	93.20	93.15	93.18	92.53	92.53	92.63	92.49
Langeland	97.46	97.54	97.44	97.44	97.58	97.49	97.58	97.43	97.45	97.44	97.42	97.36	97.71	97.89	98.17	100.00	96.31	96.29	96.28	96.40	93.70	93.43	93.34	93.29	93.18	93.12	93.12	92.52	92.52	92.54	92.47
CDC54075	96.52	96.55	96.54	96.62	96.54	96.42	96.52	96.87	96.89	96.87	97.06	96.86	96.41	96.36	96.37	96.30	100.00	99.49	99.29	96.30	93.89	93.66	93.50	93.58	93.45	93.44	93.44	92.72	92.72	92.95	92.74
CDC297	96.52	96.52	96.57	96.61	96.49	96.42	96.48	96.90	96.92	96.90	96.84	96.82	96.43	96.35	96.35	96.28	99.50	100.00	99.50	96.28	93.82	93.58	93.45	93.37	93.32	93.27	93.28	92.69	92.70	92.70	92.64
657	96.52	96.54	96.53	96.59	96.52	96.44	96.49	96.83	96.85	96.84	96.76	96.82	96.42	96.34	96.38	96.28	99.30	99.50	100.00	96.29	93.91	93.69	93.47	93.49	93.44	93.38	93.37	92.71	92.71	92.86	92.65
LochMaree	96.44	96.49	96.44	96.39	96.53	96.54	96.49	96.34	96.37	96.36	96.30	96.39	96.40	96.36	96.44	96.40	96.30	96.29	96.29	100.00	93.72	93.35	93.10	93.12	93.06	92.99	92.99	92.41	92.41	92.57	92.34
CDC1632	93.74	93.84	93.74	93.81	93.77	93.77	93.79	93.75	93.84	93.79	93.74	93.79	93.74	93.68	93.79	93.70	93.88	93.82	93.91	93.72	100.00	96.86	96.35	96.31	96.06	96.05	96.05	94.69	94.69	94.74	94.71
CDC67071	93.45	93.55	93.50	93.54	93.50	93.48	93.51	93.53	93.61	93.56	93.53	93.57	93.49	93.39	93.52	93.43	93.65	93.59	93.68	93.35	96.86	100.00	97.41	97.35	96.67	96.65	96.69	95.02	95.02	95.07	95.05
ATCC15579	93.32	93.40	93.34	93.41	93.36	93.28	93.37	93.35	93.43	93.39	93.35	93.39	93.36	93.33	93.34	93.33	93.48	93.45	93.46	93.10	96.35	97.41	100.00	99.40	97.14	97.12	97.08	95.26	95.26	95.31	95.26
AM553	93.31	93.36	93.37	93.41	93.34	93.32	93.32	93.34	93.42	93.38	93.46	93.33	93.28	93.25	93.33	93.29	93.57	93.37	93.48	93.12	96.31	97.35	99.40	100.00	97.14	97.14	97.10	95.17	95.17	95.30	95.21
AM1195	93.20	93.27	93.21	93.27	93.21	93.18	93.23	93.20	93.31	93.26	93.31	93.27	93.24	93.16	93.20	93.18	93.45	93.32	93.45	93.06	96.06	96.67	97.15	97.14	100.00	99.98	99.66	95.29	95.29	95.38	95.30
AM370	93.20	93.21	93.22	93.27	93.22	93.18	93.17	93.20	93.28	93.24	93.30	93.20	93.17	93.08	93.15	93.12	93.44	93.28	93.39	92.99	96.05	96.65	97.12	97.14	99.99	100.00	99.66	95.25	95.25	95.35	95.30
Okayama2011	93.20	93.21	93.21	93.25	93.23	93.19	93.19	93.21	93.27	93.24	93.33	93.20	93.20	93.05	93.17	93.12	93.43	93.28	93.38	93.00	96.06	96.69	97.08	97.10	99.66	99.66	100.00	95.21	95.21	95.33	95.25
NCIMB10696	92.52	92.62	92.56	92.68	92.53	92.54	92.59	92.54	92.64	92.59	92.55	92.63	92.56	92.50	92.53	92.52	92.72	92.70	92.71	92.41	94.68	95.02	95.26	95.17	95.30	95.26	95.21	100.00	99.99	99.40	99.38
DSM795	92.52	92.62	92.55	92.68	92.53	92.54	92.59	92.53	92.64	92.59	92.55	92.63	92.56	92.49	92.52	92.52	92.72	92.69	92.70	92.40	94.68	95.02	95.26	95.17	95.30	95.26	95.21	99.99	100.00	99.40	99.38
Prevot594	92.54	92.62	92.58	92.65	92.54	92.54	92.56	92.55	92.65	92.60	92.77	92.62	92.59	92.51	92.63	92.54	92.94	92.70	92.85	92.57	94.74	95.07	95.31	95.29	95.38	95.36	95.33	99.39	99.40	100.00	99.71
ATCC19404	92.54	92.56	92.57	92.65	92.55	92.54	92.54	92.56	92.63	92.59	92.58	92.56	92.52	92.45	92.49	92.46	92.73	92.65	92.65	92.34	94.71	95.06	95.26	95.20	95.30	95.30	95.25	99.38	99.38	99.71	100.00

Fig. 2.

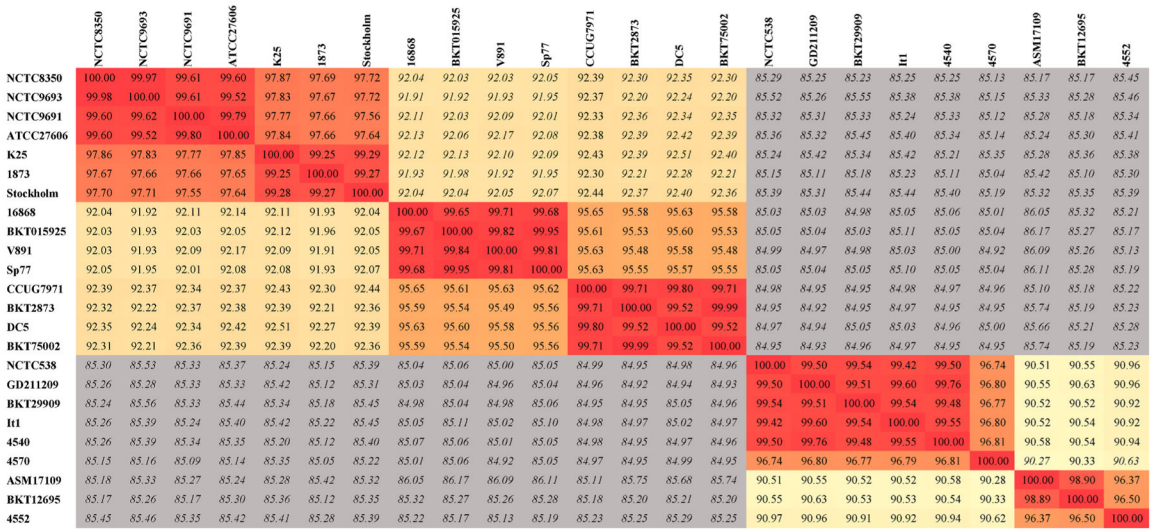
ANI matrix for metabolic Group I. Two-way ANI and coverage values were determined using an in-house script that utilizes MUMmer v.4. All pairwise comparisons within the group resulted in query coverage >70%, and ANI values delineated 2 clusters within the group, each with >95% similarity within the cluster by ANI. All pairwise ANI values in the group are >90%.



	ATCC17786	ATCC9564	CDCKA-95B	AlaskaE43	Beluga	ATCC17844	CDC4627U-1	Eklund202F	ATCC23387	Eklund17B	CDC66177
ATCC17786	100.00	99.99	99.76	99.07	97.90	94.05	94.05	94.01	94.01	93.90	93.95
ATCC9564	99.99	100.00	99.75	99.07	97.89	94.06	94.05	94.01	94.00	93.89	93.95
CDCKA-95B	99.76	99.75	100.00	99.07	97.96	94.08	94.08	94.07	94.06	93.96	93.99
AlaskaE43	99.07	99.07	99.07	100.00	97.98	94.10	94.09	94.11	94.04	93.99	93.96
Beluga	97.89	97.89	97.96	97.98	100.00	94.11	94.10	94.13	94.09	94.00	93.97
ATCC17844	94.04	94.04	94.08	94.09	94.10	100.00	99.90	99.79	99.79	97.80	97.75
CDC4627U-1	94.04	94.05	94.08	94.09	94.11	99.90	100.00	99.80	99.80	97.77	97.72
Eklund202F	94.01	94.01	94.06	94.11	94.14	99.79	99.80	100.00	99.99	97.80	97.68
ATCC23387	94.01	94.01	94.06	94.04	94.09	99.79	99.80	99.99	100.00	97.82	97.68
Eklund17B	93.89	93.89	93.96	93.99	93.99	97.79	97.77	97.80	97.81	100.00	99.11
CDC66177	93.94	93.94	93.99	93.95	93.96	97.75	97.73	97.68	97.68	99.10	100.00

Fig. 3.

ANI matrix for metabolic group II. Two-way ANI and coverage values were determined using an in-house script that utilizes MUMmer v.4. All pairwise comparisons within the group resulted in query coverage >70%, and ANI values delineated 2 clusters within the group, each with >95% similarity within the cluster by ANI. All pairwise ANI values in the group are >90%.



	NCTC8350	NCTC9693	NCTC9691	ATCC27606	K25	1873	Stockholm	16868	BKT015925	V891	Sp77	CCUG7971	BKT2873	DC5	BKT75002	NCTC538	GD211209	BKT29909	It1	4540	4570	ASM17109	BKT12695	4552
NCTC8350	100.00	99.97	99.61	99.60	97.87	97.69	97.72	*92.04*	*92.03*	*92.03*	*92.05*	92.39	*92.30*	*92.35*	*92.30*	*85.29*	*85.25*	*85.23*	*85.25*	*85.25*	*85.13*	*85.17*	*85.17*	*85.45*
NCTC9693	99.98	100.00	99.61	99.52	97.83	97.67	97.72	*91.91*	*91.92*	*91.93*	*91.95*	92.37	*92.20*	*92.24*	*92.20*	*85.52*	*85.26*	*85.55*	*85.38*	*85.38*	*85.15*	*85.33*	*85.28*	*85.46*
NCTC9691	99.60	99.62	100.00	99.79	97.77	97.66	97.56	*92.11*	*92.03*	*92.09*	*92.01*	92.33	*92.36*	*92.34*	*92.35*	*85.32*	*85.31*	*85.33*	*85.24*	*85.33*	*85.12*	*85.28*	*85.18*	*85.34*
ATCC27606	99.60	99.52	99.80	100.00	97.84	97.66	97.64	*92.13*	*92.06*	*92.17*	*92.08*	92.38	*92.39*	*92.42*	*92.39*	*85.36*	*85.32*	*85.45*	*85.40*	*85.34*	*85.14*	*85.24*	*85.30*	*85.41*
K25	97.86	97.83	97.77	97.85	100.00	99.25	99.29	*92.12*	*92.13*	*92.10*	*92.09*	92.43	*92.39*	*92.51*	*92.40*	*85.24*	*85.42*	*85.34*	*85.42*	*85.21*	*85.35*	*85.28*	*85.36*	*85.38*
1873	97.67	97.66	97.66	97.65	99.25	100.00	99.27	*91.93*	*91.98*	*91.92*	*91.95*	92.30	*92.21*	*92.28*	*92.21*	*85.15*	*85.11*	*85.18*	*85.23*	*85.11*	*85.04*	*85.42*	*85.10*	*85.30*
Stockholm	97.70	97.71	97.55	97.64	99.28	99.27	100.00	*92.04*	*92.04*	*92.05*	*92.07*	92.44	*92.37*	*92.40*	*92.36*	*85.39*	*85.31*	*85.44*	*85.44*	*85.40*	*85.19*	*85.32*	*85.35*	*85.39*
16868	92.04	91.92	92.11	92.14	92.11	91.93	92.04	100.00	99.65	99.71	99.68	95.65	95.58	95.63	95.58	*85.03*	*85.03*	*84.98*	*85.05*	*85.06*	*85.01*	*86.05*	*85.32*	*85.21*
BKT015925	92.03	91.93	92.03	92.05	92.12	91.96	92.05	99.67	100.00	99.82	99.95	95.61	95.53	95.60	95.53	*85.05*	*85.04*	*85.03*	*85.11*	*85.05*	*85.04*	*86.17*	*85.27*	*85.17*
V891	92.03	91.93	92.09	92.17	92.09	91.91	92.05	99.71	99.84	100.00	99.81	95.63	95.48	95.58	95.48	*84.99*	*84.97*	*84.98*	*85.03*	*85.00*	*84.92*	*86.09*	*85.26*	*85.13*
Sp77	92.05	91.95	92.01	92.08	92.08	91.93	92.07	99.68	99.95	99.81	100.00	95.63	95.55	95.57	95.55	*85.05*	*85.04*	*85.05*	*85.10*	*85.05*	*85.04*	*86.11*	*85.28*	*85.19*
CCUG7971	92.39	92.37	92.34	92.37	92.43	92.30	92.44	95.65	95.61	95.63	95.62	100.00	99.71	99.80	99.71	*84.98*	*84.95*	*84.95*	*84.98*	*84.97*	*84.96*	*85.10*	*85.18*	*85.22*
BKT2873	92.32	92.22	92.37	92.38	92.39	92.21	92.36	95.59	95.54	95.49	95.56	99.71	100.00	99.52	99.99	*84.95*	*84.92*	*84.95*	*84.97*	*84.95*	*84.95*	*85.74*	*85.19*	*85.23*
DC5	92.35	92.24	92.34	92.42	92.51	92.27	92.39	95.63	95.60	95.58	95.56	99.80	99.52	100.00	99.52	*84.97*	*84.94*	*85.05*	*85.03*	*84.96*	*85.00*	*85.66*	*85.21*	*85.28*
BKT75002	92.31	92.21	92.36	92.39	92.39	92.20	92.36	95.59	95.54	95.50	95.56	99.71	99.99	99.52	100.00	*84.95*	*84.93*	*84.96*	*84.97*	*84.95*	*84.95*	*85.74*	*85.19*	*85.23*
NCTC538	*85.30*	*85.53*	*85.33*	*85.37*	*85.24*	*85.15*	*85.39*	*85.04*	*85.06*	*85.00*	*85.05*	*84.99*	*84.95*	*84.98*	*84.96*	100.00	99.50	99.54	99.42	99.50	96.74	90.51	90.55	90.96
GD211209	*85.26*	*85.28*	*85.33*	*85.33*	*85.42*	*85.12*	*85.31*	*85.03*	*85.04*	*84.96*	*85.04*	*84.96*	*84.92*	*84.94*	*84.93*	99.50	100.00	99.51	99.60	99.76	96.80	90.55	90.63	90.96
BKT29909	*85.24*	*85.56*	*85.33*	*85.44*	*85.34*	*85.18*	*85.45*	*84.98*	*85.04*	*84.98*	*85.06*	*84.95*	*84.95*	*85.05*	*84.96*	99.54	99.51	100.00	99.54	99.48	96.77	90.52	90.52	90.92
It1	*85.26*	*85.39*	*85.24*	*85.40*	*85.42*	*85.22*	*85.45*	*85.05*	*85.11*	*85.02*	*85.10*	*84.98*	*84.97*	*85.02*	*84.97*	99.42	99.60	99.54	100.00	99.55	96.80	90.52	90.54	90.92
4540	*85.26*	*85.39*	*85.34*	*85.35*	*85.20*	*85.12*	*85.40*	*85.07*	*85.06*	*85.01*	*85.05*	*84.98*	*84.95*	*84.97*	*84.96*	99.50	99.76	99.48	99.55	100.00	96.81	90.58	90.54	90.94
4570	*85.15*	*85.16*	*85.09*	*85.14*	*85.35*	*85.05*	*85.22*	*85.01*	*85.06*	*84.92*	*85.05*	*84.97*	*84.95*	*84.99*	*84.95*	96.74	96.80	96.77	96.79	96.81	100.00	*90.27*	90.33	*90.63*
ASM17109	*85.18*	*85.33*	*85.27*	*85.24*	*85.28*	*85.42*	*85.32*	*86.05*	*86.17*	*86.09*	*86.11*	*85.11*	*85.75*	*85.68*	*85.74*	90.51	90.55	90.52	90.52	90.58	90.28	100.00	98.90	96.37
BKT12695	*85.17*	*85.26*	*85.17*	*85.30*	*85.36*	*85.12*	*85.35*	*85.32*	*85.27*	*85.26*	*85.28*	*85.18*	*85.20*	*85.21*	*85.20*	90.55	90.63	90.53	90.53	90.54	90.33	98.89	100.00	96.50
4552	*85.45*	*85.46*	*85.35*	*85.42*	*85.41*	*85.28*	*85.39*	*85.22*	*85.17*	*85.13*	*85.19*	*85.23*	*85.25*	*85.29*	*85.25*	90.97	90.96	90.91	90.92	90.94	90.62	96.37	96.50	100.00

Fig. 4.

ANI matrix for metabolic group III. Two-way ANI and coverage values were determined using an in-house script that utilizes MUMmer v.4. Values shaded in gray did not meet the query coverage cutoff of 70%, and thus the calculated ANI values are not accurate (values ranged between 30 and 50% coverage). This group breaks into 4 distinct clusters, each with >95% similarity within the cluster.

Table 1

Summary of BoNT-producing species of *Clostridium*, divided into phylogenetic clades.

Metabolic Group	Cluster	Species included in each clade	Reference
I	1	All *C. botulinum* type A Proteolytic *C. botulinum* type B Proteolytic *C. botulinum* type F *C. sporogenes*	(Smith et al., 2018; Weigand et al., 2015; Williamson et al., 2016; Brunt et al., 2020a)
I	2	*C. botulinum* type B *C. sporogenes*	(Weigand et al., 2015; Williamson et al., 2016; Brunt et al., 2020a)
II	3	*C. botulinum* type E Non-proteolytic, non-toxigenic *C. botulinum*	(Williamson et al., 2016; Brunt et al., 2020b)
II	4	*C. botulinum* type E Non-proteolytic *C. botulinum* type B Non-proteolytic *C. botulinum* type F Non-proteolytic, non-toxigenic *C. botulinum*	(Williamson et al., 2016; Brunt et al., 2020b)
III	5	*C. botulinum* type D *C. botulinum* type C/D *C. botulinum* type D/C	(Skarin and Segerman, 2014; Fillo et al., 2021)
III	6	*C. botulinum* type D *C. botulinum* type C *C. novyi* *C. haemolyticum*	(Skarin and Segerman, 2014; Fillo et al., 2021)
III	7	*C. botulinum* type C/D *C. novyi*	(Skarin and Segerman, 2014; Fillo et al., 2021)
III	8	*C. botulinum* type D/C *C. novyi*	(Skarin and Segerman, 2014; Fillo et al., 2021)
IV	9	*C. argentinense* (*C. botulinum* type G, *C. subterminale*, and *C. hastiforme*)	Suen et al. (1988)
N/A	10	*C. butyricum* type E	Collins and East (1998)
N/A	11	*C. baratii* type F	Collins and East (1998)