Genomic Epidemiology of *Clostridium botulinum* Isolates from Temporally Related Cases of Infant Botulism in New South Wales, Australia

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Infant botulism is a potentially life-threatening paralytic disease that can be associated with prolonged morbidity if not rapidly diagnosed and treated. Four infants were diagnosed and treated for infant botulism in NSW, Australia, between May 2011 and August 2013. Despite the temporal relationship between the cases, there was no close geographical clustering or other epidemiological links. *Clostridium botulinum* isolates, three of which produced botulism neurotoxin serotype A (BoNT/A) and one BoNT serotype B (BoNT/B), were characterized using whole-genome sequencing (WGS). *In silico* multilocus sequence typing (MLST) found that two of the BoNT/A-producing isolates shared an identical novel sequence type, ST84. The other two isolates were single-locus variants of this sequence type (ST85 and ST86). All BoNT/A-producing isolates contained the same chromosomally integrated BoNT/A2 neurotoxin gene cluster. The BoNT/B-producing isolate carried a single plasmid-borne *bont*/B gene cluster, encoding BoNT subtype B6. Single nucleotide polymorphism (SNP)-based typing results corresponded well with MLST; however, the extra resolution provided by the whole-genome SNP comparisons showed that the isolates differed from each other by >3,500 SNPs. WGS analyses indicated that the four infant botulism cases were caused by genomically distinct strains of *C. botulinum* that were unlikely to have originated from a common environmental source. The isolates did, however, cluster together, compared with international isolates, suggesting that *C. botulinum* from environmental reservoirs throughout NSW have descended from a common ancestor. Analyses showed that the high resolution of WGS provided important phylogenetic information that would not be captured by standard seven-loci MLST.

Genomic epidemiology has provided novel insights into the genetic characteristics and phylogenetic diversity of botulinum neurotoxin (BoNT)-producing *Clostridium* species (1–8). BoNT subtypes are responsible for causing the serious paralytic disease botulism and their potent neuroparalytic activities make them one of the top (tier 1) agents considered to pose a significant threat to public health if used for bioterrorism (Electronic Code of Federal Regulations—Title 42: Part 73; http://www.ecfr.gov/cgi -bin/retrieveECFR?r=PART&n=42y1.0.1.6.61) (9, 10). The same properties also make them powerful tools for both medical therapeutic and cosmetic applications (11).

Botulism is a very rare disease in Australia (National Notifiable Diseases Surveillance System [NNDSS]; http://www9.health.gov .au/cda/source/cda-index.cfm), with only 20 cases reported since 1991. However, a global survey found that Australia had one of the highest numbers of notified cases of infant botulism in the world (12, 13). Infant botulism results from the ingestion of *Clostridium botulinum* spores which germinate and temporarily colonize the infant's colon, followed by growth of vegetative cells that produce BoNT (14–16). This form of the disease only occurs in infants, generally under 1 year old, as they have relatively low levels of gastric acid and poorly developed normal gut microflora (17). It is epidemiologically distinct from food-borne botulism which results from the ingestion of preformed BoNT from contaminated food (18) (http://www.cdc.gov/ncidod/dbmd /diseaseinfo/files/botulism.PDF).

The first clinical signs in infant botulism are usually constipation and lethargy, with progression to poor feeding, muscular weakness, abnormal eye movements, and, in the absence of intervention, progressive symmetrical flaccid paralysis and respiratory arrest due to neuromuscular junction blockage (19, 20). A number of risk factors have been identified, including the consumption of honey (21–24), herbal infusions (25), contamination of infant food such as powdered formula (26), and exposure to household dust (27) and disrupted soil (20, 28). Contact with pet terrapins has also recently been identified as a risk factor for infant botulism caused by *Clostridium butyricum* producing BoNT/E (29). However, sources of infection or specific risk factors have generally not been identified for either sporadic or clustered cases (30). Infant botulism has a low mortality rate but can be associated with significant morbidity. However, the timely ad-

ministration of human botulism immune globulin intravenous (BIG-IV) has been shown to reduce morbidity, as evidenced by a significant reduction in the mean length of the hospital stay (19, 20, 28, 31, 32).

The diagnosis of infant botulism is confirmed by isolation of *C. botulinum* from the stool, followed by confirmation of toxin production by the mouse neutralization assay (33). Emerging evidence suggests that the genotyping of strains can help to link clustered cases and identify potential shared sources of infections (34–38). Unfortunately, the resolution of standard typing methods and the frequent absence of environmental samples often make it difficult to conclusively link cases or identify environmental reservoirs. The use of whole-genome sequencing (WGS) to compare entire bacterial genomes at the single nucleotide level can, however, greatly improve clustering of bacterial pathogens (39).

There are seven well-characterized BoNT serotypes (A to G), and a potential eighth type was recently reported (H) (40, 41) but is still to be confirmed. The neurotoxins are produced by four genetically and physiologically distinct groups of *C. botulinum* (groups I to IV) (42, 43) or less frequently by *Clostridium butyricum* or *Clostridium baratii* (44–47). The different serotypes of toxin can cause neuromuscular paralysis of significantly different durations and are further classified into subtypes based on *bont* gene sequence variations. The majority of human botulism cases are caused by *C. botulinum* group II, nonproteolytic strains that produce toxin serotypes B, E, or F or by *C. botulinum* group I, proteolytic strains that produce toxins A, B, or F. Group I strains are closely related to nontoxogenic *Clostridium sporogenes* isolates (7, 37, 48). Some strains are bivalent and express more than one type of BoNT (e.g., serotype BoNT/Ab, whereby the uppercase letter denotes the dominant toxin serotype) (2). Some BoNT/A strains also carry a silent serotype B toxin, with the presence of the inactive toxin indicated in parentheses [e.g., BoNT/A(B)] (2). BoNT is produced by neurotoxin gene complexes that are located on mobile genetic elements (MGE) which can move horizontally between strains with diverse genetic backgrounds, making the *bont* gene or BoNT complex sequences unreliable targets for phylogenetic comparisons (35, 49, 50).

The largest epidemiological studies of infant botulism have been carried out in Japan, where 31 cases have been reported since 1986 (4), and in California in the United States, where 978 cases have been reported since 1976, with 20 to 50 cases reported each year (34). Dabritz et al. applied amplified fragment length polymorphism (AFLP) analysis to genotype isolates and BoNT-specific real-time PCR to characterize BoNT subtypes. Comparative analyses identified several distinct clades associated with spatiotemporal clusters or clusters containing both infant and environmental (i.e., honey) isolates, implicating a common-source exposure (34). Considerable diversity was documented in the genomic backgrounds of isolates expressing the same toxin subtypes, indicating extensive horizontal transfer of neurotoxin gene clusters (34). The most in-depth genomic characterization of *C. botulinum* isolates causing infant botulism was carried out on all isolates from reported cases of infant botulism in Japan between 2006 and 2011 (4). A comparative genomic analysis of the 10 sequenced isolates and 13 reference sequences available in GenBank revealed that only ~33% of the *C. botulinum* genomes represented a core genome, reflecting the large phylogenetic distances separating lineages of *C. botulinum* (49). Core genome SNP analysis was shown to improve cluster resolution, separating the largest group of serotype A(B) isolates into two distinct lineages (4).

Currently, there is no information about the genomic characteristics of *C. botulinum* in Australia. Therefore, WGS was used to perform comparative genomic analyses on *C. botulinum* isolates from a potential cluster of four infant botulism cases that occurred in NSW within a 16-month period.

MATERIALS AND METHODS

Case reports and isolate characterization. In Australia, clinical cases of infant botulism are rare with only six cases notified in NSW in the last 15 years. Four of these cases were reported between September 2011 and August 2013. These four cases were documented in infants born at term after uncomplicated pregnancies. All were admitted to the intensive care units of pediatric referral hospitals in Sydney and required tracheal intubation. Infant botulism was suspected following characteristic electromyography features. Only two cases were treated with human botulism immune globulin intravenous (BIG-IV) (19), and all recovered. Public health investigations were not able to identify epidemiologic links between the cases, and no risk factors or potential sources of infection were identified. The clinical courses of the cases are summarized in Table 1, and clinical reports on cases 1 and 2 have been previously published (31).

C. botulinum was isolated from stool samples of all cases. Isolates were cultured on botulism selective medium and identified using API 20A (bio-Mérieux, Hazelwood, MO), Rapid ID 32A (Remel, Lenexa, KS), and 16S rRNA gene sequencing. A mouse bioassay was used to confirm the presence of *C. botulinum* neurotoxin and to identify the toxin serotype (33). Monovalent neutralizing antitoxins were supplied by the Centers for Disease Control and Prevention, Atlanta, GA.

DNA extraction and WGS. Genomic DNA was extracted from pure cultures using the DNeasy blood and tissue kit (Qiagen), and 200-bp fragment libraries were prepared using the Ion Xpress fragment library kit and Ion Xpress barcode adapters, following the protocol for 100-ng DNA input library preparation (Life Technologies, USA). Samples were then pooled and sequenced together on an Ion 318 Chip in an Ion Torrent PGM, using the Ion PGM template OT2 200 kit and an Ion PGM 200 sequencing kit (Life Technologies, USA).

Bioinformatic analyses. Sequence data were processed and analyzed using Torrent Suite 4.0.2. Sequencing reads were mapped to the reference genomes, *C. botulinum* ATCC 3502 A1 (GenBank accession number AM412317.1), *C. botulinum* A2 str. Kyoto (GenBank accession number NC_012563.1), and *C. botulinum* B1 str. Okra (GenBank accession number CP000939.1), using the Torrent Alignment plug-in v4.0-r77189. Variants were detected with the variantCaller plug-in v4.0-r76860, using default settings: positions used to call single nucleotide polymorphisms (SNPs) had to have a minimum Phred-scaled call quality of ≥10, a minimum read fold coverage of ≥6, and a maximum strand bias of 0.95. *De novo* assembly was also performed on sequencing reads using Assembler plug-in v3.4.2.0, which uses MIRA v3.9.9 development to assemble contigs. Contigs were then further assembled into scaffolds using Mauve v2.3.1 (51). WebACT (http://www.webact.org/WebACT/home) was used to produce BLAST comparisons of sequenced isolates against each other and the reference genome and plasmid sequences.

Seven-loci multiple locus sequence typing (MLST) (35) was performed *in silico*, by uploading *de novo*-assembled contig files to the PubMLST *Clostridium botulinum* query page (http://pubmlst.org/cbotulinum/). The MLST allele sequences obtained from NSW1_A2 were then downloaded and concatenated to create an MLST pseudomolecule (see Table S1 in the supplemental material). Reads from all four strains were aligned to this pseudomolecule to confirm the allele sequences, which were then submitted to the PubMLST *Clostridium botulinum* sequence query page to confirm the allele profiles. MLST allele profiles were then compared to the list of allele profiles available for download from PubMLST, and a phylogenetic tree was created using the PubMLST tree drawing tool.

De novo-assembled contigs were also uploaded to CSI Phylogeny 1.0a

TABLE 1 Clinical information relating to the four cases of infant botulism

Case (isolate name)	Toxin	Background	Mo/yr	Location in NSW	Age at presentation (wks)	Duration of intubation (days)	Duration of ICU[a] admission (days)	Duration of hospital admission (days)	BIG-IV[b]
1 (NSW1_A2)	A	Exclusive breastfeeding Semirural living Recent renovation of residence	May 2011	Bathurst	10	45	53	72	Not given
2 (NSW2_A2)	A	Formula feeds Urban living	September 2011	Sydney	8	7	7	23	Not given
3 (NSW3_A2)	A	Formula feeds Urban living; had visited farm	March 2013	Sydney	19	13	23	25	Given on day 5 of illness
4 (NSW4_B6)	B	Exclusive breastfeeding Urban living	August 2013	Sydney	27	14	17	30	Given on day 6 of illness

[a] ICU, intensive care unit.
[b] BIG-IV, human botulism immune globulin intravenous.

(https://cge.cbs.dtu.dk/services/CSIPhylogeny/), a webserver which identifies SNPs from whole-genome sequencing data, filters and validates the SNP positions, and then infers phylogeny based on concatenated SNP profiles (52). *C. botulinum* ATCC 3502 was used as the reference strain. SNPs were excluded if they were in regions with a minimum fold coverage of <10, within 10 bp of another SNP or <15 bp from the end of a contig. The analysis also included isolates belonging to each of the five lineages of *C. botulinum* group I strains, recently described by Gonzalez-Escalona et al. (3).

BoNT-complex sequences were extracted from *de novo*-assembled contigs and compared to publically available BoNT cluster sequences using BLASTn. Phylogenetic analyses of BoNT gene sequences were performed using MEGA6. Multiple sequence alignments were performed using MUSCLE, and phylogenetic analyses were performed using the maximum likelihood method based on the Kimura 2 parameter model (53, 54). The accession numbers of BoNT gene sequences used to construct phylogenetic trees are listed in Table S2 in the supplemental material.

Nucleotide sequence accession number. The genomic data have been deposited in the NCBI Sequence Read Archive (SRA) (http://www.ncbi.nlm.nih.gov/Traces/sra/) under accession number PRJNA273428.

RESULTS AND DISCUSSION

Three of the *C. botulinum* isolates were confirmed to produce toxin A (NSW1_A2, NSW2_A2, and NSW3_A2) and one (NSW4_B6) was confirmed to produce toxin B in the mouse bioassay. Sequence reads from NSW1_A2, NSW2_A2, and NSW3_A2 were mapped to reference genome sequences of *C. botulinum* ATCC 3502 A1 and *C. botulinum* A2 str. Kyoto, and reads from NSW4_B6 were mapped to *C. botulinum* B1 str. Okra and *C. botulinum* A2 str. Kyoto. Read mapping and single nucleotide variant (SNV) detection metrics (see Table S3 in the supplemental material) showed that all four NSW isolates were highly diverse in comparison to the reference strains. All isolates were more similar to *C. botulinum* A2 str. Kyoto than to their other respective reference strains, but there were still high levels of sequence divergence, with reads from BoNT/A-carrying strains covering only approximately 88 to 90% of the *C. botulinum* A2 str. Kyoto genome and containing >40,000 variants and reads from the BoNT/B-carrying isolate covering approximately 89% of the genome and containing >37,000 variants.

In silico MLST analysis revealed that two of the BoNT/A-carrying isolates shared the same MLST profile (*aroE*-11, *mdh*-5, *aceK*-11, *oppB*-9, *rpoB*-8, *recA*-6, *hsp*-8), which represented a new sequence type (ST) that was a 6-locus match to both the ST10 and ST53 allele profiles. This profile has been submitted to the PubMLST *C. botulinum* database and designated ST84. NSW2_A2 contained a new *mdh* allele sequence, which was submitted to the PubMLST *C. botulinum* database and named *mdh*-30; the resulting ST profile (*aroE*-11, *mdh*-30, *aceK*-11, *oppB*-9, *rpoB*-8, *recA*-6, *hsp*-8) was designated ST85. The BoNT/B-carrying isolate also had a new ST (*aroE*-13, *mdh*-5, *aceK*-11, *oppB*-9, *rpoB*-8, *recA*-6, *hsp*-8), which was a 6-locus match to ST84 and a 5-locus match to ST10, ST8 and ST15, and ST53 (Fig. 1). Because all four of the NSW infant botulism isolates share at least five loci in common, they are likely to belong to the same clonal lineage (55).

Isolate relatedness was further examined by whole-genome SNP comparisons. The CSI Phylogeny webserver was used to identify SNPs separating the four NSW infant botulism isolates from one another and from selected published genome sequences available in NCBI GenBank (Fig. 2). The SNP distance matrix comparison genomes are included in Table S5 in the supplemental material. The topology of this tree closely resembled that of the MLST-based phylogenetic tree (see Fig. S1 in the supplemental
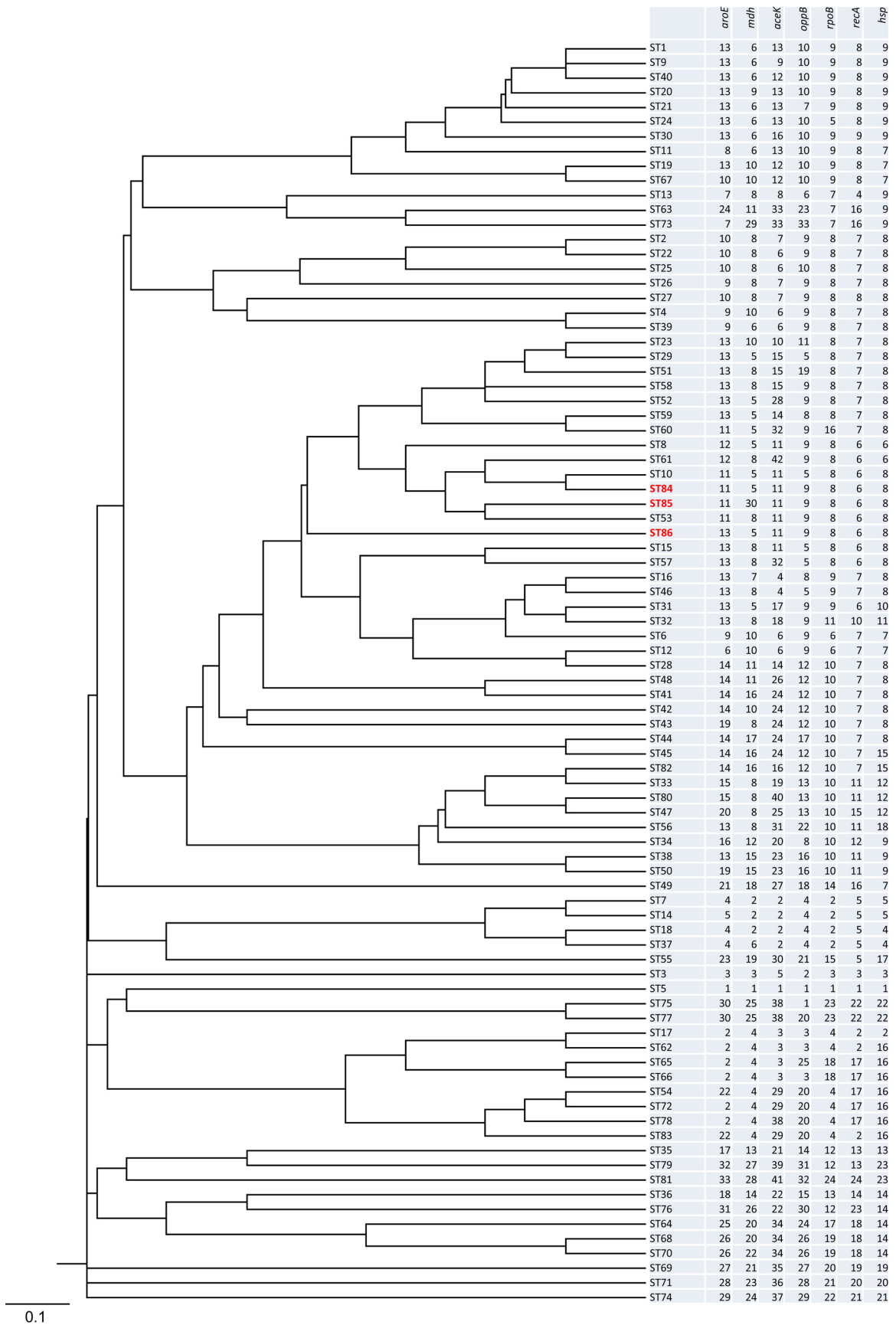
FIG 1 MLST-based phylogenetic tree of NSW infant botulism isolates. The positions of the new ST84 represented by NSW1_A2 and NSW3_A2 (*aroE*-11, *mdh*-5, *aceK*-11, *oppB*-9, *rpoB*-8, *recA*-6, *hsp*-8), the new ST85 represented by NSW3_A2 (*aroE*-11, *mdh*-30, *aceK*-11, *oppB*-9, *rpoB*-8, *recA*-6, *hsp*-8), and the new ST86 represented by NSW4_B6 (*aroE*-13, *mdh*-5, *aceK*-11, *oppB*-9, *rpoB*-8, *recA*-6, *hsp*-8) are indicated. The corresponding 7-locus allele profiles used to create the tree are shown on the right. Branch lengths indicate the linkage distance between isolates calculated with the PubMLST tree drawing tool.
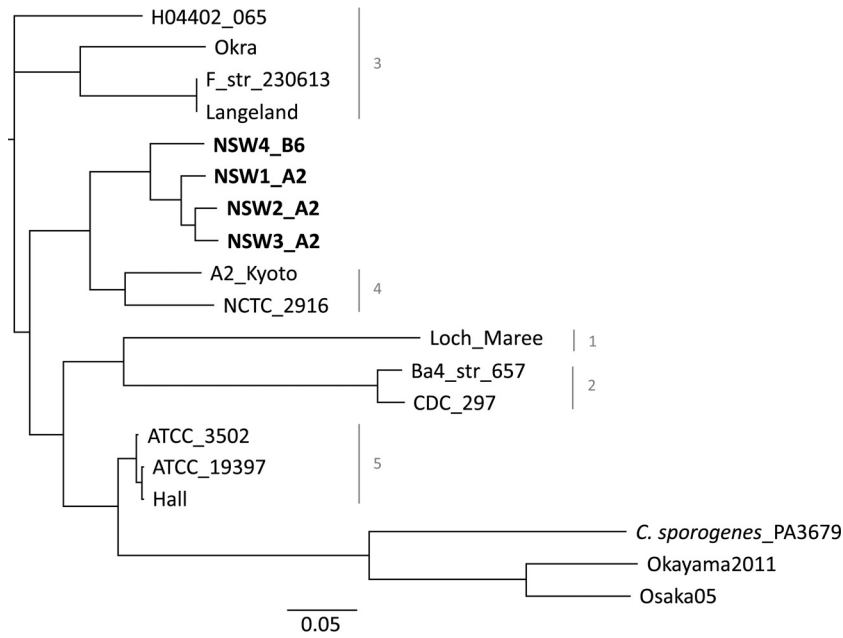
FIG 2 SNP-based phylogenetic tree of NSW infant botulism isolates and selected *C. botulinum* genome sequences from GenBank. SNP detection and phylogenetic inference were performed by the CSI Phylogeny webserver (52). Branch lengths correspond to numbers of nucleotide substitutions per site. The clustering of published isolates corresponded well with the lineage groupings 1 to 5 of *C. botulinum* group I strains reported by Gonzalez-Escalona et al. (3), which are indicated in gray. Isolates sequenced in this study and the Japanese infant botulism isolates Osaka05 and Okayama2011 did not cluster with any of these previously described lineages.

material), with the four NSW isolates clustering together. The SNP difference matrix, calculated by CSI Phylogeny, showed that *bont*/A-carrying isolates harbored >3,500 SNP differences compared to one another and >8,000 SNP differences compared to the *bont*/B-carrying isolate. These numbers are not likely to reflect

the exact numbers of SNPs between isolates as some may belong to repetitive or poorly aligned regions that can interfere with SNP detection, and there will be a certain amount of bias introduced by the use of a reference genome for variant detection, as regions found in our isolates, but not present in *C. botulinum* A2 Str.
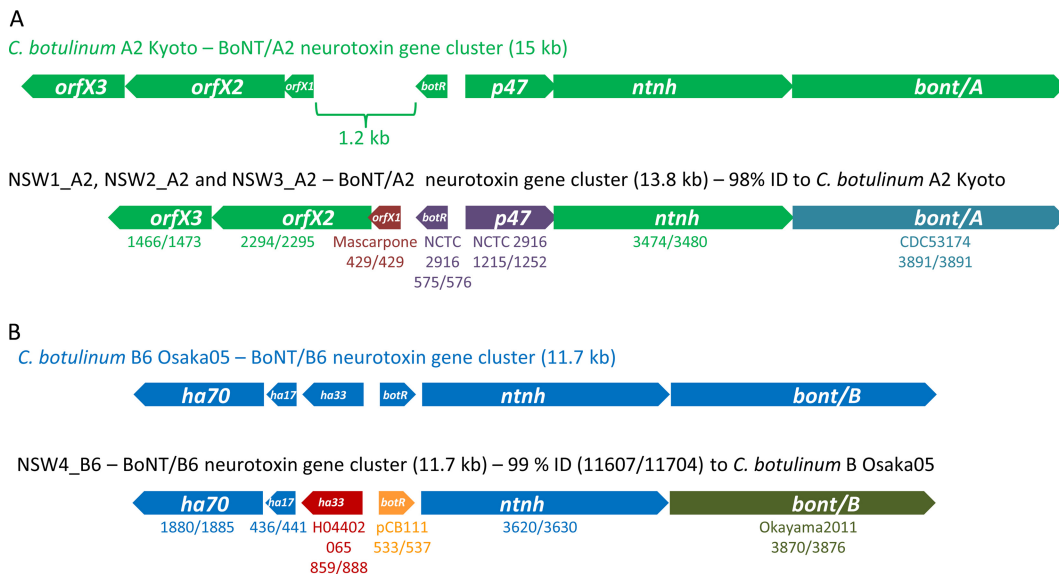
FIG 3 Structure of *bont* gene clusters from NSW infant botulism isolates. (A) Comparison of the *bont*/A2 cluster from *C. botulinum* A2 str. Kyoto and the three *bont*/A2-carrying NSW infant botulism isolates. All isolates had identical *bont* complex sequences apart from NSW1_A2, which had a single nucleotide difference in *orfX1*. All isolates had the 1.2-kb deletion between *orfX1* and *botR* that is also present in *C. botulinum* strain Mascarpone (GenBank accession number DQ310546.1) and *C. botulinum* strain CDC41370 (GenBank accession number FJ981696.1). (B) Comparison of the *bont*/B cluster from *C. botulinum* strain Osaka05 and the *bont*/B-carrying NSW infant botulism isolate. The closest BLASTn percent identity hit for the whole gene cluster is indicated above the cluster schematic diagram, and the number of identical nucleotide hits to the closest BLASTn match for each gene is shown beneath; for open reading frames (ORFs) where the closest match was not the reference *bont* cluster, the corresponding strain name is given.

Kyoto, will be not be included in the analysis. However, a second variant comparison, using CLC Genomics Workbench, confirmed that all isolates were separated from one another by at least 4,000 SNPs and that the SNPs were distributed across the entire genome (see Fig. S2 in the supplemental material). This suggests that even though these strains are likely to have descended from a common clonal ancestor, they are not sufficiently related to one another to indicate that any of the cases had been infected from the same environmental source of *C. botulinum* spores.

All *bont*/A-carrying isolates had a single chromosomally encoded BoNT/A2 neurotoxin cluster integrated into the *arsC* operon integration site (49) (see Fig. S3 in the supplemental material). Multiple sequence alignments showed that the 13,786-bp sequences of all three BoNT/A2 neurotoxin clusters were identical, apart from one single nucleotide difference in strain NSW1_A2 (data not shown). The synonymous SNP was in *orfX1* at nucleotide position 105 (C>T). The high degree of homology suggested that the neurotoxin gene cluster was horizontally acquired by all three isolates from a common donor source. Extensive horizontal gene transfer of toxin clusters among strains from diverse clonal lineages is thought to be responsible for the lack of phylogenetic correlation between the toxin subtype and the genomic background (38, 49, 56–58). This BoNT/A2 neurotoxin cluster lacked a 1.2-kb insertion between *botR* and *orfX1* that is typically present in A2 toxin gene clusters. The absence of this insertion was previously described in the BoNT/A2 neurotoxin cluster of *C. botulinum* type A str. Mascarpone (58). Other regions of the cluster shared higher nucleotide identity levels with other diverse *bont*/A2 cluster gene sequences from the BLASTn database (Fig. 3). Table S6 in the supplemental material shows the locations of SNPs detected when reads from NSW1_A2, NSW2_A2, and NSW3_A2 are mapped to the *bont* cluster region of *C. botulinum* type A str. Mascarpone (GenBank accession number DQ310546.1). The BoNT/B-producing isolate contained a single plasmid-encoded neurotoxin gene cluster that shared 99% identity with the *bont*/B6 cluster from *C. botulinum* B str. Osaka05 (59) (Fig. 3; see also Fig. S3 in the supplemental material). The plasmid containing the *bont*/B cluster in NSW4_B6 (pNSW4_B6) appears to be similar in size and structure to plasmid pCB111 (GenBank accession number AB855771.1) from *C. botulinum* B str. 111 (60) (see Fig. S3 in the supplemental material), which encodes a BoNT/B2 subtype toxin.

bont toxin gene phylogenies showed that the identical *bont*/A gene sequences from the BoNT/A-producing strains fell within the A2 subtype group, which contains the neurotoxin from *C. botulinum* A2 str. Kyoto that was isolated from a case of infant botulism in Kyoto, Japan, in 1978 (61) (Fig. 4A). The *bont*/B gene sequence fell within the B6 subtype cluster together with the BoNT/B-encoding gene from *C. botulinum* B str. Osaka05, isolated from a case of infant botulism in Osaka, Japan, in 2005 (59) and the BoNT/B-encoding gene from *C. botulinum* B str. Okayama2011 isolated from a case of infant botulism in Okayama, Japan, in 2011 (4) (Fig. 4B). Isolate NSW4_B6 from NSW is the first strain identified outside Japan to carry a BoNT subtype B6. There were four SNP differences between Osaka05 and Okayama2011 (4), and eight and six SNP differences between NSW4_B6 and Osaka05 and Okayama2011, respectively. Table S7 in the supplemental material shows the locations of the SNPs detected when reads from NSW4_B6 were mapped to the *bont* cluster region of *C. botulinum* B str. Osaka05. Strains Osaka05 and
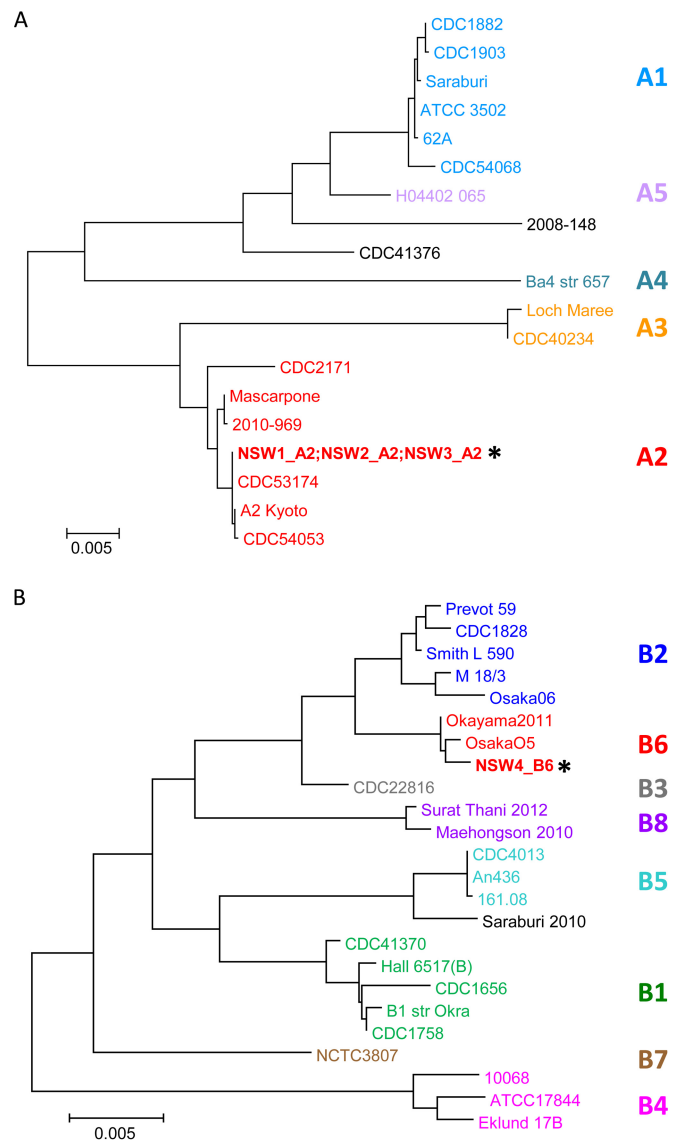

FIG 4 *bont* gene phylogenetic analyses. (A) Dendrogram of selected *bont*/A nucleotide sequences. The *bont*/A sequences from NSW1_A2, NSW2_A2, and NSW3_A2 clustered with *bont*/A2 gene sequences. (B) Dendrogram of selected *bont*/B genes, showing the NSW4_B6 clustered with *bont*/B6 sequences. The neurotoxin subtypes are indicated to the right of their corresponding color-coded branches. Branch lengths represent the number of substitutions per site as indicated by the bars.

Okayama2011 were previously shown to belong to a phylogenetically distinct *C. botulinum* group I clade, based on approximately 50% of their core genome SNP markers not matching those of any of the other analyzed strains (4). In Fig. 2, these isolates were more closely related to *C. sporogenes* PA3679 (48) than to other group I *C. botulinum* isolates and were distantly removed from NSW4_B6, which clustered with the *bont*/A2-carrying NSW isolates. These observations indicated that *bont*/B6-carrying plasmids may be acquired by phylogenetically diverse *C. botulinum* isolates from different geographical areas.

High levels of similarity between *bont* gene sequences of isolates from infant botulism cases in NSW and those from cases of infant botulism in Japan give further weight to evidence indicating

that different BoNT subtypes have differing toxicogenic and/or immunogenic properties, leading to different disease etiologies (43, 56). *C. botulinum* spores are ubiquitous in the environment and have been isolated from soil, marine, and fresh water samples from most parts of the world (13). Genomic analyses suggest that diverse populations of clonally related nontoxigenic clostridia are present throughout different regions of NSW. The acquisition of MGE-containing specific BoNT clusters could then influence these isolates to cause infant botulism. However, further analyses of environmental and both infant botulism and food-borne botulism isolates need to be carried out to confirm this.

In conclusion, this study reports the first genomic characterization of *C. botulism* isolates from Australia. Whole-genome SNP-based analyses revealed that isolates from four temporally related cases of infant botulism belonged to a separate WGS clade of *C. botulinum* group I isolates that did not cluster with strains from any of five previously defined lineages (3) or core genome SNP-based clades (1) of published isolates. By standard MLST and *bont* gene sequencing, two of the three *bont*/A-carrying isolates would have appeared identical, indicating that they could have belonged to an epidemiologically related cluster. However, the high resolution power of WGS revealed that all strains were separated from one another by at least 3,500 SNPs. There have been no in-depth studies investigating rates of SNP accumulation over time in *C. botulinum*. However, based on estimated molecular clock rates for other bacterial pathogens, the numbers of SNPs separating the four NSW isolates indicated that they had probably diverged from their most recent common ancestor at least hundreds of years ago (62). Conversely, the presence of only one SNP among the three BoNT/A2 cluster sequences indicated that this genomic region had been more recently acquired by all three isolates, potentially from the same donor source.

ACKNOWLEDGMENTS

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