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Positive Regulation of Botulinum Neurotoxin Gene Expression by CodY in *Clostridium botulinum* ATCC 3502

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Botulinum neurotoxin, produced mainly by the spore-forming bacterium *Clostridium botulinum*, is the most poisonous biological substance known. Here, we show that CodY, a global regulator conserved in low-G+C Gram-positive bacteria, positively regulates the botulinum neurotoxin gene expression. Inactivation of *codY* resulted in decreased expression of *botA*, encoding the neurotoxin, as well as in reduced neurotoxin synthesis. Complementation of the *codY* mutation in *trans* rescued neurotoxin synthesis, and overexpression of *codY* in *trans* caused elevated neurotoxin production. Recombinant CodY was found to bind to a 30-bp region containing the *botA* transcription start site, suggesting regulation of the neurotoxin gene transcription through direct interaction. GTP enhanced the binding affinity of CodY to the *botA* promoter, suggesting that CodY-dependent neurotoxin regulation is associated with nutritional status.

Clostridium botulinum is a Gram-positive, spore-forming anaerobic bacterium that produces botulinum neurotoxin, which is the most poisonous biological substance known to mankind. Botulinum neurotoxin blocks neurotransmission in cholinergic nerves (1, 2) in humans and animals to cause botulism, a potentially lethal flaccid paralysis. Despite its extreme toxicity, botulinum neurotoxin is widely utilized as a powerful therapeutic agent to treat numerous neurological disorders (3, 4).

Seven antigenically distinct botulinum neurotoxin types (A to G), and several subtypes therein, have been identified (5–9). Moreover, a novel toxin type H was recently proposed (10) and awaits further characterization (11). Type A1 neurotoxin is well characterized as a consequence both of its frequent involvement in human botulism worldwide and of its use as a therapeutic agent (12). Type A1 neurotoxin is produced as a complex containing the neurotoxin itself and associated nontoxic proteins (ANTPs) that comprise a nontoxic nonhemagglutinin protein (NTNH) and three hemagglutinin proteins (HAs; HA17, HA33, and HA70) (13–15). The NTNH protects the neurotoxin from low pH- and protease-induced inactivation in the gastrointestinal tract (16), while the HAs assist the neurotoxin absorption, probably by interacting with oligosaccharides and E-cadherin on intestinal epithelial cells (17).

In *C. botulinum* type A1, the genes encoding the neurotoxin (*botA*) and ANTPs (*ntnh*, *ha17*, *ha33*, *ha70*) are located in a gene cluster and are organized in two operons, namely, the *ntnh-botA* and *ha* operons (18). Within the neurotoxin gene cluster, *botR*, located between the two operons, encodes an alternative sigma factor that is a member of group 5 of the sigma 70 family, including *Clostridium difficile* TcdR, *Clostridium perfringens* UviA, and *Clostridium tetani* TetR. BotR directly controls the transcription of both the *ntnh-botA* and *ha* operons (19, 20). An Agr-like quorum sensing system was found to be involved in positive regulation of the neurotoxin production (21), suggesting that the cell density-dependent signals control neurotoxin production. Also, the CLC_1093/CLC_1094, CLC_1914/CLC_1913, and CLC_0661/CLC_0663 two-component signal transduction systems (TCSs) were proposed to positively regulate the neurotoxin synthesis (22). The first report on negative regulation of neurotoxin synthesis demonstrated the CBO0787/CBO0786 TCS to repress neurotoxin synthesis by the CBO0786 response regulator directly binding to the conserved −10 site of the core promoter of *ntnh-botA* and *ha* operons and blocking BotR-directed transcription (23).

Botulinum neurotoxin gene transcription is growth phase dependent. Transcription of the neurotoxin gene cluster is increased in the exponential growth phase, peaks at the transition from late-exponential- to early-stationary-phase cultures, and is drastically decreased during the stationary phase (24–26). Botulinum neurotoxin production is also affected by the availability of certain carbon and nitrogen sources (27–29). These findings suggest that botulinum neurotoxin synthesis is controlled through nutrition-related metabolic pathways. Although the metabolic and regulatory networks of pathogenic bacteria are only partly understood, the transition state regulator CodY has been shown to be an important regulatory link between metabolism and virulence factor synthesis in many low-G+C Gram-positive pathogens (30, 31). In *Bacillus subtilis*, CodY controls the expression of over 100 genes by sensing the level of GTP and branched-chain amino acids (BCAAs), thereby governing the adaptation of the cell to the transition from exponential growth to stationary phase (32–34). As a highly conserved global regulator, CodY not only shows the common role in metabolic regulation in other low-G+C Gram-positive bacteria but also controls virulence gene expression in *Clostridium difficile* (35, 36), *Clostridium perfringens* (37), *Bacillus cereus* (38), *Bacillus anthracis* (39), *Staphylococcus aureus* (40), *Streptococcus pneumoniae* (41), *Streptococcus pyogenes* (42), *Streptococcus mutans* (43), and *Listeria monocytogenes* (44).

Here, we evaluated the role of CodY in the regulation of botulinum neurotoxin synthesis. Genetic data suggest that CodY plays

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a positive regulatory role in botulinum neurotoxin gene transcription and neurotoxin production. Biochemical evidence suggests that CodY interacts with a 30-bp region in the promoter of *botA*.

MATERIALS AND METHODS

Strains, plasmids, oligonucleotides, and culture. Bacterial strains and plasmids used in this study are listed in Table S1 in the supplemental material, and all oligonucleotide primers are listed in Table S2 in the supplemental material. *C. botulinum* group I type A1 strain ATCC 3502 (45) and the derived *codY* mutant were grown in anaerobic tryptone-peptone-glucose-yeast extract (TPGY) medium at 37°C in an anaerobic workstation with an atmosphere of 85% N_2, 10% CO_2, and 5% H_2 (MK III; Don Whitley Scientific Ltd., Shipley, United Kingdom). Cell counts were determined using the three-tube most-probable-number (MPN) method. *Escherichia coli* conjugation donor CA434 (46), *E. coli* NEB 5-alpha strain (New England BioLabs, Ipswich, MA), and *E. coli* LMG 194 (Life Technologies, Carlsbad, CA) were grown in Luria-Bertani (LB) medium at 37°C. When appropriate, growth media were supplemented with 100 μg/ml ampicillin, 50 μg/ml kanamycin, 25 μg/ml chloramphenicol, 250 μg/ml cycloserine, 15 μg/ml thiamphenicol, or 2.5 μg/ml erythromycin.

Construction of mutants. An insertional inactivation mutation in *codY* in *C. botulinum* ATCC 3502 was constructed using the ClosTron system (47) (kindly provided by Nigel P. Minton, University of Nottingham, Nottingham, United Kingdom). The intron was targeted between nucleotides 526 to 527 in the antisense strand of the *codY* sequence. The designed retargeted mutagenesis plasmid pMTL007C-E2::*codY* was ordered from DNA 2.0 (Menlo Park, CA). Plasmid retargeting and mutant selection were carried out as previously described (47). PCR was performed to confirm the integration of the Ll.LtrB-derived intron in the desired site using primers *codY*-F and *codY*-R.

For complementation and overexpression, a 1,265-bp fragment encompassing *codY* and the 5′ noncoding region, including the putative promoter, was amplified using primers *codY*-82151-F and *codY*-82151-R. The amplified DNA was digested with NotI and NheI and cloned into the pMTL82151 vector (48) to create pMTL82151::*codY*. pMTL82151::*codY*, or pMTL82151, was transferred to the *C. botulinum* ATCC 3502 wild-type (WT) strain or the *codY* mutant by conjugation from *E. coli* CA434 to generate the *codY*-pMTL82151::*codY* complementation strain, the WT-pMTL82151::*codY* overexpression strain, and the WT-pMTL82151 and *codY*-pMTL82151 control strains.

Southern blotting. Genomic DNA from the ATCC 3502 wild-type strain and *codY* mutant and the pMTL007C-E2 plasmid DNA were digested overnight with HindIII (New England BioLabs) and subjected to Southern blot analysis with the Ll.LtrB-derived intron-specific probe as previously described (23).

Western blotting. One milliliter of late-exponential cultures of the ATCC 3502 wild type and the *codY* mutant was centrifuged, and the pellet was analyzed for CodY by Western blotting using a rabbit polyclonal antiserum against *B. subtilis* CodY (kindly provided by Abraham L. Sonenshein, Tufts University, Boston, MA) and IRDye 800-labeled goat anti-rabbit IgG secondary antibody (LI-COR Biosciences, Lincoln, NE) and visualized using the LI-COR Odyssey infrared imaging system.

RNA isolation, cDNA synthesis, and quantitative reverse transcription-PCR (RT-qPCR). Total RNA from *C. botulinum* ATCC 3502 and the *codY* mutant was isolated using the RNeasy minikit (Qiagen, Hilden, Germany) and treated with the RNase-free DNase set (Qiagen) and the DNA-free kit (Ambion, Austin, TX), as previously described (49). The RNA concentration was determined using the NanoDrop ND1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA). The integrity of RNA was evaluated with the Agilent Technologies 2100 bioanalyzer (Agilent Technologies, Santa Clara, CA).

cDNA samples were prepared from 800 ng of RNA using the DyNAmo cDNA synthesis kit (Thermo Fisher Scientific). Real-time RT-qPCR was carried out with 16S *rrn* as the reference gene (50), in the Rotor-Gene 3000 real-time thermal cycler (Qiagen). The reaction mixtures were composed of 1× Maxima SYBR green qPCR master mix (Thermo Fisher Scientific), 0.5 μM each primer, and 4 μl of 10^2-fold (*botA*) or 10^5-fold (16S *rrn*) diluted cDNA template in a total volume of 25 μl. Cycling conditions included 10 min at 95°C and 40 cycles of 95°C for 15 s and 60°C for 60 s. PCR efficiencies were determined for each primer pair based on a standard curve made from serial dilutions of pooled cDNA. The calculated efficiencies were 0.93 for 16S *rrn* and 0.97 for *botA*. Melting curve analysis was performed immediately after PCR to confirm specificity of the PCR amplification products. All reactions were performed with three biological replicates, each with two technical replications. Target gene expression was normalized to the 16S *rrn* transcript level using a comparative threshold cycle (C_T) method (51). All data were calibrated against the transcript levels in the wild-type cells collected at 5 h of incubation.

Neurotoxin ELISA. Aliquots (1 ml) of culture supernatants were collected at time points ranging from 5 to 96 h by centrifugation at 15,000 × *g* for 5 min and tested for botulinum neurotoxin using a commercial type A neurotoxin enzyme-linked immunosorbent assay (ELISA) kit (Tetracore, Rockville, MD) according to the manufacturer's instruction. The absorbance was measured at 405 nm on a microtiter plate reader (Multiskan Ascent, Thermo Fisher Scientific). For each ELISA plate, a standard curve was generated using purified type A neurotoxin (kindly provided by Michel R. Popoff, Institute Pasteur, Paris, France), and all the coefficients of determination (R^2) were above 0.997. According to the linear range of the neurotoxin standard curve, the culture supernatants were diluted from 1:20 to 1:6,000 with ELISA blocking buffer and subjected to ELISA.

Expression and purification of recombinant CodY. To construct the plasmids for the expression of N-terminal 6-histidine translation fusion to CodY, a PCR product was generated using the primers *codY*-30-F and *codY*-30-R. The PCR product was digested with EcoRI and SphI and cloned into plasmid pBAD30 (kindly provided by Bruno Dupuy, Institute Pasteur). The resultant plasmid was transformed into *E. coli* strain LMG194 (Life Technologies).

Protein expression was induced with 0.2% arabinose at 37°C for 8 h. Cells from a 200-ml culture were collected, resuspended in 10 ml of lysis/binding buffer (500 mM NaCl, 20 mM imidazole, 20 mM Tris-HCl [pH 7.9]), and lysed by sonication. The lysate was centrifuged at 10,000 × *g* for 15 min and filtered through a 0.45-μm-pore-size filter. The lysate was loaded with 1 ml of Novagen His bind affinity resin (EMD Millipore, Billerica, MA) and then washed by 10 ml of lysis/binding buffer and 20 ml of wash buffer (500 mM NaCl, 60 mM imidazole, 20 mM Tris-HCl [pH 7.9]). The bound protein was removed with 4 ml of elution buffer (500 mM NaCl, 500 mM imidazole, 20 mM Tris-HCl [pH 7.9]). Eluted proteins were examined by SDS-PAGE prior to dialysis using a Novagen D-tube dialyzer against 500 ml of dialysis buffer (300 mM NaCl, 20% glycerol, 50 mM Tris-HCl [pH 8.0]) overnight at 4°C. Protein concentrations were determined using the Bradford assay (Bio-Rad, Hercules, CA) with bovine serum albumin (Sigma-Aldrich, St. Louis, MO) as a standard.

EMSA. A 262-bp fragment (P*ntnh-botA* probe) comprising the upstream region of the *ntnh-botA* operon (bp −210 to bp 52 of *ntnh*) was amplified by PCR (23) using 5′ IRDye 700 (LI-COR)-labeled primers P*ntnh-botA*-F and P*ntnh-botA*-R (IDTDNA, Coralville, IA). Electrophoretic mobility shift assay (EMSA) was performed with 1 nM IRDye 700-labeled P*ntnh-botA* probe or similarly labeled control probe (49), 0 to 1 μM recombinant CodY, 1 μg of poly(dI-dC) (Thermo Fisher Scientific), 2.5% glycerol, and 5 mM $MgCl_2$ in binding buffer (10 mM Tris, 50 mM KCl, 1 mM dithiothreitol [DTT] [pH 7.8]). When specified, 2 mM GTP or 10 mM (each) isoleucine, leucine, and valine (BCAA) was added. For competition assays, a 200-fold molar excess of unlabeled probe was added. Binding reactions were allowed to proceed for 20 min at room temperature and then resolved on a 5% native polyacrylamide gel run in 0.5× Tris-borate-EDTA (TBE) at 4°C for 1 h at 110 V.

DNase I footprinting. DNase I footprinting was performed in multiple replicates using a modification described in reference 52. The P*ntnh-botA* probe was amplified using 6-carboxyfluorescein (FAM)-labeled for-

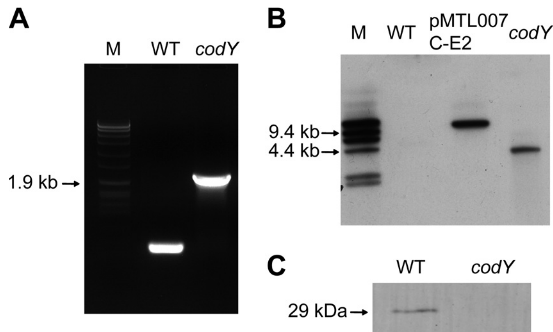

FIG 1 Insertional inactivation of *codY* in the *Clostridium botulinum* ATCC 3502 strain. (A) PCR analysis of *codY* mutation. Ll.LtrB-derived intron insertion was detected with primers flanking the insertional site yielding a 2.2-kb product in the *codY* mutant. M, Roche DNA molecular weight marker VII. (B) Southern blot analysis of HindIII-digested DNA from the WT, the pMTL007-C-E2 plasmid, and the *codY* mutant with intron-specific probe. (C) Western blot analysis of CodY expression in WT and *codY* mutant cultures.

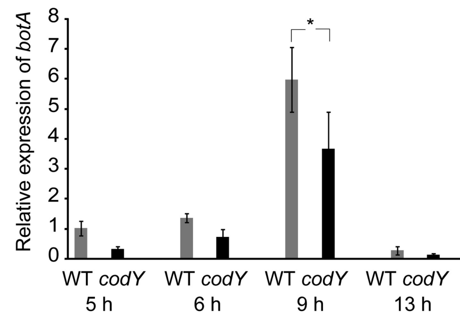

FIG 2 Expression of *botA* in the *Clostridium botulinum* ATCC 3502 wild-type strain and the *codY* mutant. RNA was isolated after 5, 6 (mid-exponential phase), 9 (transition phase), and 13 (early stationary phase) h of growth and analyzed for relative *botA* expression using RT-qPCR. Target gene expression was normalized to 16S *rrn* and calibrated to the WT at 5 h. Error bars represent standard deviations from three biological replicates. *, $P < 0.05$ (Student's *t* test).

ward primer and HEX-labeled reverse primer as previously described (23). Binding reactions were performed as described for EMSA except that 2 mM GTP, 10 nM 5′-FAM-labeled probe, and 5 μM the CodY protein were used. Binding reactions were allowed to proceed for 20 min at room temperature prior to digestion using 0.002 to 0.2 Kunitz units of DNase I (Sigma-Aldrich) for 5 min. Reactions were stopped by the addition of 22 μl of 0.5 M EDTA and heated at 70°C for 10 min. The digested DNA fragments were purified with the QIAquick PCR purification kit (Qiagen) and separated on the Applied Biosystems 3730xl DNA analyzer with GeneScan LIZ-500 internal size standard (Applied Biosystems, Foster City, CA). The electropherograms were analyzed using the Peak Scanner software (Applied Biosystems).

RESULTS

Inactivation of *codY*. To confirm successful mutation of *codY* in *C. botulinum* group I type A1 strain ATCC 3502, the mutant DNA was screened by PCR using primers flanking the mutation target site. A 400-bp PCR product was amplified from the ATCC 3502 wild-type (WT) DNA, while the *codY* mutant DNA yielded a 2.2-kb amplification product demonstrating integration of a 1.8-kb fragment of the Ll.LtrB-derived intron, containing an erythromycin resistance gene (*ermB*), into the *codY* coding region (Fig. 1A). Consecutive cultures showed the *codY* mutant to be erythromycin resistant and stable. A single insertion of the Ll. LtrB-derived intron into the genome of the *codY* mutant was confirmed by Southern blotting with a probe specific for the Ll.LtrB-derived intron (Fig. 1B). Expression of CodY was analyzed using Western blotting with antibodies raised against *B. subtilis* CodY (32). In contrast to the WT, the mutant failed to express CodY (Fig. 1C). Finally, a slightly lower optical density at 600 nm (OD_{600}) was observed for the *codY* mutant than for the WT at the transition between late exponential and early stationary growth phases (see Fig. S1 in the supplemental material). However, equal cell counts (2.3×10^8/ml) measured for both cultures at 11 h suggested that inactivation of *codY* did not essentially affect growth.

CodY positively regulates botulinum neurotoxin gene expression. To test whether the inactivation of *codY* affects the transcription of the neurotoxin gene, the relative expression levels of *botA* was compared between the WT and the *codY* mutant at four time points during exponential and early stationary growth phases using RT-qPCR. Maximum *botA* transcript levels were observed at the transition between late exponential and early stationary growth phases (9 h) in both strains (Fig. 2), suggesting that the tightly regulated neurotoxin expression pattern was maintained despite *codY* mutation. The transcript levels of *botA* in the *codY* mutant were half of those observed in the WT, with most prominent differences being observed at 9 h of growth (Fig. 2).

To investigate whether the neurotoxin production is affected by the *codY* mutation, the amounts of neurotoxin in the culture supernatants of the WT and the *codY* mutant were determined in cultures grown for 5 to 96 h (24) by using ELISA. As expected, an increasing amount of neurotoxin was present in all culture supernatants collected in the exponential and late stationary growth phases (Fig. 3). In the WT culture supernatant, the neurotoxin concentrations peaked at 60 μg/ml at 48 h of growth, whereas the toxin concentrations in the *codY* mutant culture supernatant reached only 30 μg/ml at 48 h of growth. The half-reduced neurotoxin levels in the *codY* mutant compared to those in the WT were consistent with observations at the transcriptional level.

The *codY* mutation was complemented by introducing pMTL82151::*codY*, containing the *codY* coding sequence and its putative native promoter, into the *codY* mutant. The resulting *codY*-pMTL82151::*codY* strain did not show significant growth difference from the WT-pMTL82151 and *codY*-pMTL82151 vector control strains (see Fig. S2 in the supplemental material) but exhibited greater expression of CodY than the WT-pMTL82151strain (Fig. 4). The neurotoxin concentrations in the culture supernatant of the *codY*-pMTL82151::*codY* strain reached 3- and 2-fold-higher levels than in the *codY*-pMTL82151 control at 48 and 96 h of growth, respectively (Fig. 5), suggesting that complementation of the *codY* mutation rescued neurotoxin production. Moreover, the complementation resulted in significantly increased neurotoxin production in relation to that of the WT, probably because pMTL82151::*codY* replicated at a high copy number in the *codY* mutant and led to induced expression of the plasmid-encoded *codY*. To test this hypothesis, a *codY* overexpression strain was constructed by introducing pMTL82151::*codY* into the WT, generating the WT-pMTL82151::*codY* strain. No significant growth differences were observed between the WT-pMTL82151::*codY* overexpression strain, the *codY*-pMTL82151::*codY* complementation strain, and the WT-pMTL82151 and

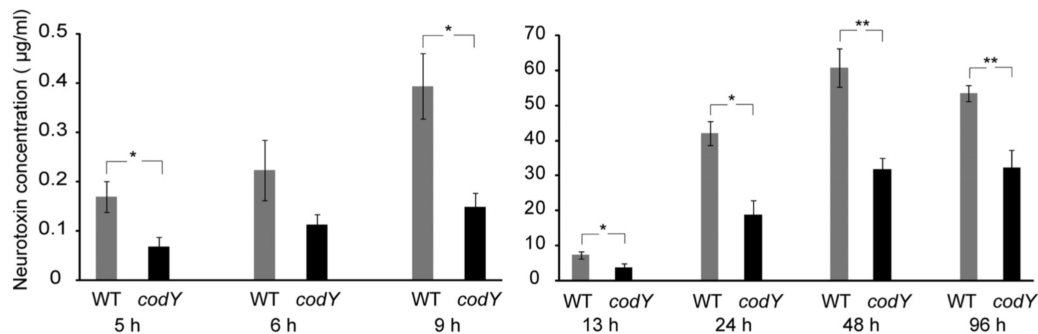

FIG 3 Neurotoxin production by *Clostridium botulinum* ATCC 3502 wild-type strain and *codY* mutant. ELISA analysis of botulinum neurotoxin in WT and *codY* mutant culture supernatants after 5, 6, and 9 h (left) and 13, 24, 48, and 96 h (right) of growth. Error bars indicate standard deviations from three biological replicates. *, $P < 0.05$; **, $P < 0.01$ (Student's *t* test).

codY-pMTL82151 vector control strains (see Fig. S2 in the supplemental material). Similar to the observations in complementation, Western blotting also showed that the WT-pMTL82151::*codY* strain expressed CodY at a considerably higher level than the WT-pMTL82151 control (Fig. 4). Expectedly, the neurotoxin concentration in the culture supernatant of the WT-pMTL82151::*codY* strain was also significantly higher than that in the WT-pMTL82151 supernatant (Fig. 5).

Taken together, these data suggest that CodY plays a positive regulatory role in transcription of the botulinum neurotoxin gene and neurotoxin production.

CodY interacts with the botulinum neurotoxin gene promoter. To investigate whether CodY regulates the transcription of the neurotoxin gene cluster directly, an EMSA was performed to examine the binding of recombinant CodY to a probe encompassing the upstream region of *ntnh* containing the promoter of the *ntnh-botA* operon (P*ntnh-botA* probe) (23). The presence of an increasing concentration of CodY caused a shift in the mobility of the P*ntnh-botA* probe (Fig. 6A), and the specific nature of binding was further confirmed by disappearance of both protein-DNA complexes using competition with a 200-fold excess of unlabeled probe. To test if CodY of *C. botulinum* responded to GTP and BCAAs, reported to enhance the binding affinity of CodY to its target gene promoters in some low-G+C Gram-positive bacteria (35, 43, 53–55), the EMSA procedure was repeated with the addition of 2 mM GTP or 10 mM BCAAs in the binding reactions. When 2 mM GTP was present, an enhanced binding affinity of CodY was observed with the P*ntnh-botA* probe (Fig. 6B). In contrast, the presence of 10 mM BCAAs did not enhance the binding affinity of CodY to the P*ntnh-botA* probe (Fig. 6C). A 16S *rrn* fragment (P16S *rrn* probe), serving as a negative control, did not show a significant shift with increased concentration of CodY (Fig. 6D). These results suggest that CodY recognizes and binds to the promoter of the *ntnh-botA* operon *in vitro*. GTP, but not BCAAs, may enhance the binding affinity of CodY to the promoter of the *ntnh-botA* operon, supporting the hypothesis that CodY acts in response to the intracellular GTP level in *C. botulinum*.

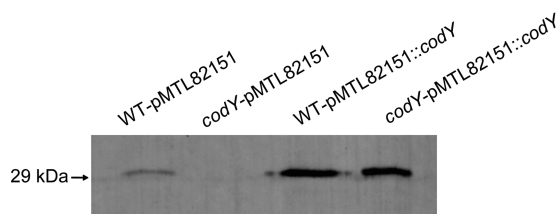

FIG 4 CodY expression in the complementation and overexpression strains. Western analysis of CodY expression in the *codY*-pMTL82151::*codY* complementation strain, WT-pMTL82151::*codY* overexpression strain, and WT-pMTL82151 and *codY*-pMTL82151 vector control strains.

To further identify the CodY-binding site, a DNase I footprinting analysis with multiple replicates was performed using fluorescently end-labeled P*ntnh-botA* probe. In both strands of the P*ntnh-botA* sequence, a 30-bp region (bp −108 to −79 upstream of *ntnh*), encompassing the transcriptional start site of the *ntnh-botA* operon (20), was consistently found to be protected by CodY from DNase I digestion (Fig. 7A and B). Analysis of the DNA sequence of the 30-bp protection region yielded a putative CodY-binding motif, AATaTaCTGAAAAaT, with three mismatches (lowercase) to the proposed consensus CodY-binding motif, AATTTTCWGAAAATT, reported for *B. subtilis* (56) and *Lactococcus lactis* (57, 58), and with similarity to the CodY-binding sites in *C. difficile* (36) and in *S. aureus* (43). Another putative CodY-binding motif, tATTTTtAaAAAATT, similarly containing three mismatches (lowercase) to the consensus motif, was present in the probe in an AT-rich region neighboring the core promoter −35 region of the *ntnh-botA* operon (Fig. 7C). Immediately upstream of the 30-bp protection region, the core promoter of the *ntnh-botA* operon (20) and the AT-rich region showed a weak interaction with CodY with the sense strand, but no interaction with the antisense strand, of the P*ntnh-botA* probe (Fig. 7A and B). The results suggest that CodY interacts mainly with a 30-bp region in the promoter region of the *ntnh-botA* operon.

DISCUSSION

We suggest that CodY positively regulates botulinum neurotoxin expression in *C. botulinum* group I type A1 strain ATCC 3502. Positive regulation was supported by genetic and biochemical lines of evidence and adds to the slowly growing body of information on neurotoxin regulation in *C. botulinum* and, more generally, virulence regulation in clostridia. Direct regulation was supported by CodY interacting with a 30-bp region encompassing the transcriptional start site in the promoter region of the *ntnh-botA* operon (20). Analysis of the 30-bp region indicated the presence of a putative CodY-binding motif, AATaTaCTGAAAAaT, with three mismatches (lowercase) to the consensus CodY-binding motif, AATTTTCWGAAAATT (56–58). The DNA-binding domain at the C terminus of CodY is highly conserved in low-G+C

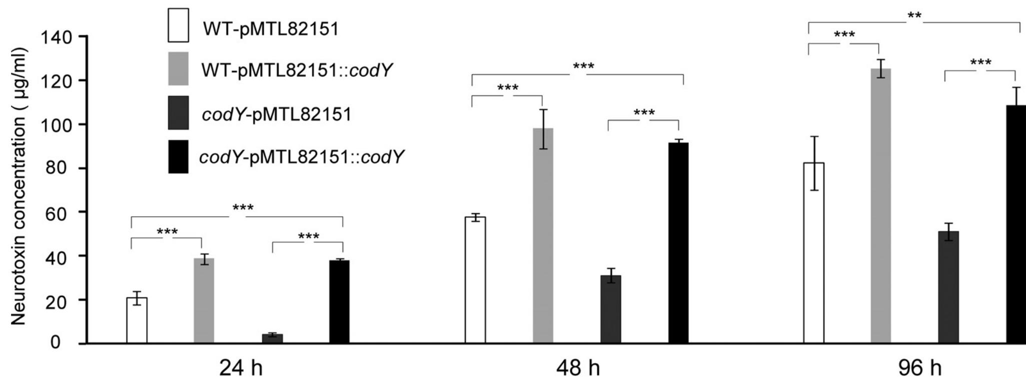

FIG 5 Neurotoxin production by the complementation and overexpression strains. ELISA analysis of botulinum neurotoxin in culture supernatants of the *codY*-pMTL82151::*codY* complementation strain, WT-pMTL82151::*codY* overexpression strain, and WT-pMTL82151 and *codY*-pMTL82151 vector control strains. Error bars represent standard deviations from three biological replicates. **, $P < 0.01$; ***, $P < 0.001$ (one-way ANOVA with Tukey's *post hoc* test).

Gram-positive bacteria (59, 60), supplying the CodY homologs with a general property in the recognition of target DNA. However, the degenerate sequence AATTTTCWGAAAATT is probably not a reliable guide for identifying the actual CodY-binding motif in *C. botulinum*. CodY has been reported to conduct its physiological function through interaction with sequences possessing up to five mismatches to the consensus motif (56, 61). The AT count of the P*ntnh-botA* probe is higher than 75%, which facilitates the presence of several putative CodY-binding motifs with four or five mismatches to the consensus CodY-binding motif AATTTTCWGAAAATT. However, all putative CodY-binding motifs, except the one within the 30-bp region of P*ntnh-botA*, showed no or little interaction with CodY in ATCC 3502. These findings suggest that CodY regulates botulinum neurotoxin gene expression mainly through interaction with the 30-bp region in the promoter of the *ntnh-botA* operon. The weak interaction of CodY with the putative CodY-binding motifs in the AT-rich region and other sites in the sense strand of P*ntnh-botA*, if at all physiologically relevant, is unlikely to represent a major mode of CodY-dependent regulation.

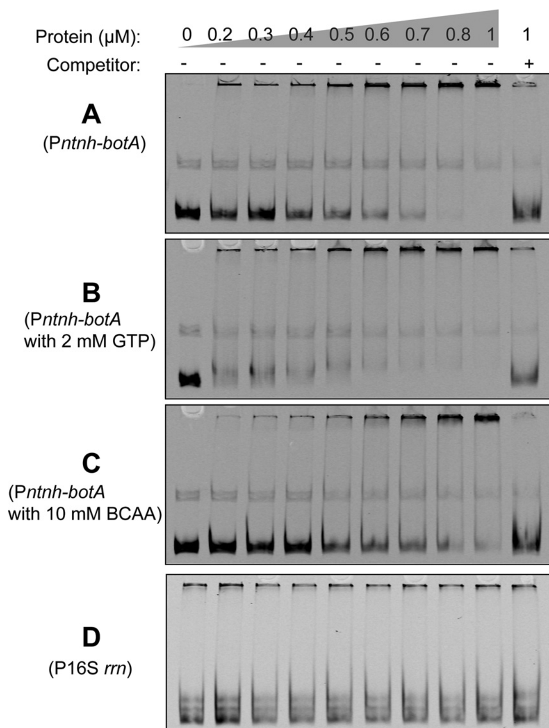

FIG 6 Electrophoretic mobility shift assay (EMSA) for binding of CodY to the promoter of *ntnh-botA* operon. P*ntnh-botA* probe was incubated with increasing concentrations of CodY in the absence of effector (A) or with 2 mM GTP (B) or 10 mM (each) isoleucine, leucine, and valine (branched-chain amino acids [BCAAs]) (C). Specificity was confirmed using 200-fold molar excess of unlabeled competitor DNA. (D) CodY did not show significant binding to P16S *rrn* probe.

Immediately upstream of the proposed 30-bp CodY-binding region lies the core promoter −10 site of the *ntnh-botA* operon. The alternative sigma factor BotR specifically recognizes the core promoter and directs the RNA polymerase to transcribe the *ntnh-botA* operon (20). The close vicinity of the core promoter and the identified CodY-binding site raises the question of whether CodY interacts with BotR and/or the RNA polymerase core enzyme, thereby enhancing the transcription of the *ntnh-botA* operon. Another interesting question is if CodY interacts with the CBO0786 TCS response regulator, a repressor shown by us to specifically bind to the core promoter −10 region (23), thereby derepressing the transcription of the *ntnh-botA* operon. Interestingly, two putative CodY-binding motifs, AATTTTCAGtAgATa and AATTTTgTtAAAATa, each with three mismatches (lowercase) to the consensus CodY-binding motif, are found upstream of the translation start site of *cbo0787*, encoding the cognate TCS sensor kinase, implying that CodY might play a physiological function in regulating the *cbo0787* expression. Further studies on the interaction of CodY with the two-component signal transduction system CBO0787/CBO0786 may offer new insights into the mechanisms controlling botulinum neurotoxin synthesis.

To facilitate the adaptation of the bacterial cell to different nutrient environments, CodY regulates multiple cellular activities by monitoring the intracellular level of GTP, BCAA, or both (32, 53, 62). The fact that GTP enhanced CodY binding to the P*ntnh-botA* probe suggests that CodY-mediated regulation of botulinum neurotoxin synthesis is associated with nutrition status. While availability of glucose is known to induce botulinum neurotoxin formation (27), several amino acids, such as arginine, proline, and glutamate, have been suggested to repress it (28, 29). Further characterization of the CodY regulon in *C. botulinum* is required to

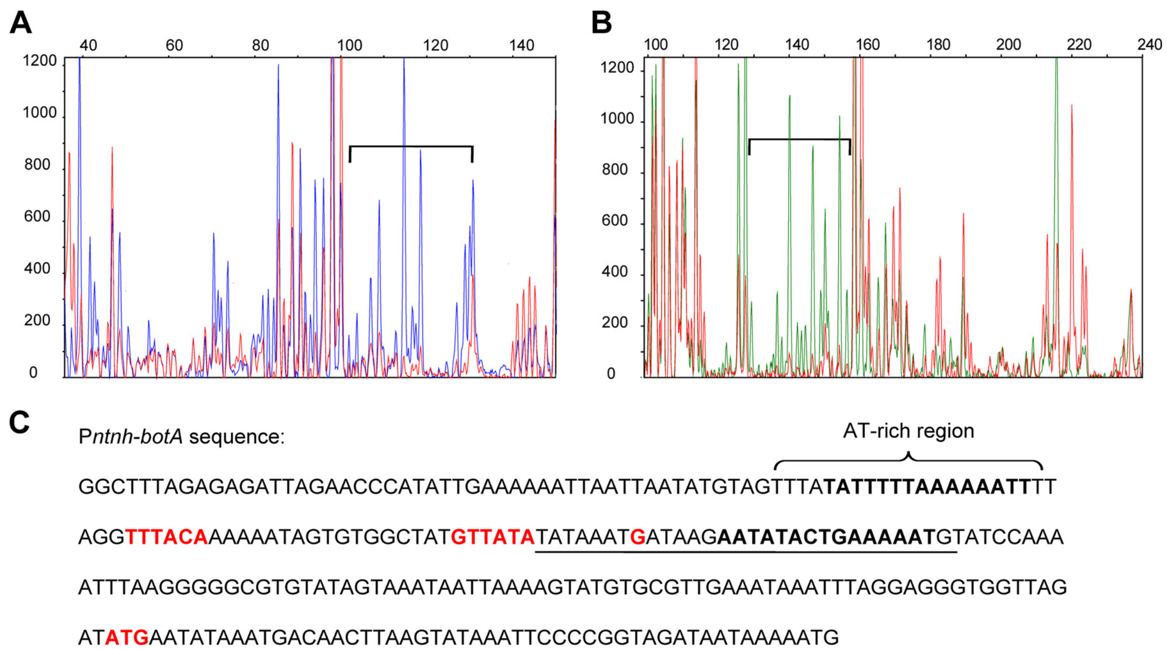

FIG 7 DNase I footprinting assay for binding of CodY to the promoter of the *ntnh-botA* operon. Fragment analysis of the 5′-FAM-labeled sense strand (A) and the 5′-HEX-labeled antisense strand (B) of the P*ntnh-botA* probe shows DNase I digestion in the absence of (blue peaks in panel A and green peaks in panel B) or with (red peaks) 5 μM CodY. The protection region is indicated by square brackets in electropherograms, and the corresponding sequence is underlined in the P*ntnh-botA* sequence (C). Two putative CodY-binding motifs are indicated in bold letters. The core promoter −10 and −35 regions, transcriptional start site, and translational start site of the *ntnh-botA* operon are shown in red.

understand the relationship between cellular metabolism and botulinum neurotoxin synthesis. In many other low-G+C Gram-positive species, CodY represses the transcription of genes accounting for approximately 5% of the genome, including synthesis of several amino acids (BCAAs, histidine, and arginine) and transport of amino acids, peptides, and sugars (34). In addition to repressory effects, CodY-mediated induction has been recognized for carbon overflow metabolism. Under nutrient-rich conditions, *B. subtilis* uses CodY to activate the conversion of pyruvate derived from glycolysis to excreted overflow products such as acetate, lactate, and acetoin (34). In clostridia, pyruvate is metabolized by multiple anaerobic fermentation pathways and converted into a variety of fermentation end products, such as lactate, acetate, acetone, ethanol, butyrate, and butanol (49, 63, 64). Considering the temporal overlap between botulinum neurotoxin gene expression in batch cultures of *C. botulinum* and the pH-dependent switch from acidogenic to solventogenic metabolism described in batch cultures of *Clostridium acetobutylicum* (65), it is tempting to speculate that (CodY-mediated) botulinum neurotoxin production is linked to pyruvate metabolism.

The role of CodY in virulence regulation has also been documented in other clostridial pathogens. In *C. perfringens* type D strain CN3178, CodY was shown to positively regulate the epsilon toxin gene expression (37). Interestingly, epsilon toxin production was additionally dependent on the Agr quorum sensing system (66) also proposed to positively control neurotoxin synthesis in *C. botulinum* (21). As opposed to positive control of toxin production in *C. perfringens* and *C. botulinum*, CodY-mediated repression of *C. difficile* enterotoxin A (TcdA) and cytotoxin B (TcdB) production through transcriptional inactivation of *tcdR* under nutrient-rich conditions is well established (35, 36). TcdR is an alternative sigma factor that directs the transcription of *tcdA* and *tcdB* (67) and is both structurally and functionally closely related to BotR (68). Whether CodY exerts a regulatory action on *botR* in *C. botulinum* remains to be elucidated.

In the present study, we quantified neurotoxin production in *C. botulinum* ATCC 3502 using a commercial ELISA with type A neurotoxin as a standard. Our results suggest a 2- to 10-fold-higher neurotoxin production by *C. botulinum* ATCC 3502 than previously reported for *C. botulinum* type A strains 62A, Hall A-*hyper*, and NCTC 2916 with an in-house-constructed ELISA (24). Variation in neurotoxin titers between different *C. botulinum* strains, stocks, and culture media is recognized by laboratories working with this pathogen (24, 69) and can be poorly explained by the fragmented knowledge currently available for neurotoxin regulation. Systematic research efforts on the environmental cues and cellular mechanisms regulating neurotoxin production in different *C. botulinum* strains with various genetic systems encoding the neurotoxins is therefore warranted.

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