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Relative Neurotoxin Gene Expression in *Clostridium botulinum* Type B, Determined Using Quantitative Reverse Transcription-PCR

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A quantitative reverse transcription-PCR (qRT-PCR) method was developed to monitor the relative expression of the type B botulinum neurotoxin (BoNT/B) gene (*cntB*) in *Clostridium botulinum*. The levels of *cntB* mRNA in five type B strains were accurately monitored by using primers specific for *cntB* and for the reference gene encoding the 16S rRNA. The patterns and relative expression of *cntB* were different in the different strains. Except for one of the strains investigated, an increase in *cntB* expression was observed when the bacteria entered the early stationary growth phase. In the proteolytic strain *C. botulinum* ATCC 7949, the level of *cntB* mRNA was four- to fivefold higher than the corresponding levels in the other strains. This was confirmed when we quantified the production of extracellular BoNT/B by an enzyme-linked immunosorbent assay and measured the toxicity of BoNT/B by a mouse bioassay. When the effect of exposure to air on *cntB* expression was investigated, no decline in the relative expression was observed in spite of an 83% reduction in the viable count based on the initial cell number. Instead, the level of *cntB* mRNA remained the same. When there was an increase in the sodium nitrite concentration, the bacteria needed a longer adjustment time in the medium before exponential growth occurred. In addition, there was a reduction in the expression of *cntB* compared to the expression of the 16S rRNA gene at higher sodium nitrite concentrations. This was most obvious in the late exponential growth phase, but at the highest sodium nitrite concentration investigated, 45 ppm, a one- to threefold decline in the *cntB* mRNA level was observed in all growth phases.

Clostridium botulinum is an obligately anaerobic, endospore-forming bacterium which produces characteristic neurotoxins. The botulinum neurotoxins (BoNTs) are among the most potent biological toxins that have been identified in nature and can cause fatal neuroparalytic conditions known as botulism (13). Four of the seven different serotypes of BoNTs (types A to G), types A, B, E, and F, are the types reported to cause food-borne botulism in humans.

In the food industry novel food products are constantly being developed by using new formulations, new technologies, or new packaging systems. The development of a new product may result in an increased risk of botulism, if the risk is not properly addressed. In particular, a refrigerated processed food with extended durability may represent a severe food-borne poisoning hazard due to heat treatment at a lower temperature and the anaerobic atmosphere provided by the packaging (25). Recent surveys in Sweden to determine the prevalence of *C. botulinum* in slaughtered pigs and cattle revealed a high occurrence (62 and 73%, respectively) of type B spores in fecal samples from these animals (5, 6). This high occurrence results in a potential risk of contamination of the meat during slaughtering.

Classical food-borne botulism is primarily due to the ingestion of preformed neurotoxin in food. Little is known about the direct regulation of the BoNT gene and how different environ-

mental factors relevant to foods affect *cnt* gene expression. In previous studies workers relied on the in vivo mouse bioassay for determining the levels of BoNT in the samples (18, 24). However, the mouse bioassay is an indirect measure of toxin concentration; i.e., the effect of exposure to active toxin is measured, which does not necessarily reflect the actual expression of *cnt* and/or the production of BoNT, and the results can therefore give a false picture of what is really happening at the regulatory level in the bacterial cell. Recently, a regulatory gene encoding an approximately 21-kDa protein, *botR*, was identified in the *cnt* clusters (29), and it has been shown that this gene acts as a positive regulator in *C. botulinum* type A (19, 20).

In recent years, in vitro methods have been developed for monitoring *cnt* expression in *C. botulinum*; these methods include a gene reporter system (7) and competitive reverse transcription (RT)-PCR (21). However, conventional PCR is not suitable for quantification as the final concentration of PCR products is not linearly related to the initial target nucleic acid concentration (23). Introduction of real-time PCR technology has made accurate quantification of RNA and DNA possible (4, 11, 15).

In the present study, a quantitative RT-PCR (qRT-PCR) method was developed to monitor and determine the level of *cntB* mRNA in *C. botulinum* type B. Specific fluorogenic probes were designed for *cntB* and for the housekeeping gene encoding the 16S rRNA (*rrn*). Recently, the 16S rRNA gene has been used as a reference gene for quantification of the mRNA transcript of the germination gene, *gerA*, in group I *C. botulinum* type B and *Clostridium sporogenes* (2). The amounts of *cntB* mRNA that accumulated at various times were deter-

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TABLE 1. Sequences and fluorescent dye of primers and TaqMan probes used for qRT-PCR

Target gene	GenBank accession no.	Primer or probe	Nucleotide positions ^a	Nucleotide sequence ^b	Reference
<i>cntB</i>	M81186	fBn	723–746	5'-AAAGTAGATGATTTACCAATTGTA-3'	5
		rBn	916–938	5'-GTTAGGATCTGATATGCAAATA-3'	5
		cntBprobe	888–913	5'-ACCTTGTTAAGTCTATCAACTATCCC-3'	This study
<i>rm</i>	X68173	f16S	991–1012	5'-GTGTCGTGAGATGTTGGGTTAA-3'	This study
		r16S	1178–1197	5'-TAGCTCCACCTCGCGGTATT-3'	This study
		16Sprobe	1014–1035	5'-TCCCGCAACGAGCGCAACCCTT-3'	This study

^a The positions correspond to the nucleotide positions downstream from the ATG start codon of the corresponding *C. botulinum* genes.

^b The TaqMan probes were constructed with the 5' reporter dye 6-carboxyfluorescein and an internal quencher, Dark Quencher, located at the position indicated by underlining.

mined for both proteolytic and nonproteolytic strains of *C. botulinum* type B during growth. Furthermore, the levels of *cntB* mRNA were correlated with the production of extracellular type B botulinum neurotoxin (BoNT/B) (i.e., the total amount of inactive toxin and the biologically active toxin) by using an enzyme-linked immunosorbent assay (ELISA) and with the toxicity of exposure to the active toxin by using a mouse bioassay. Finally, qRT-PCR was used to quantify the changes in *cntB* mRNA levels in *C. botulinum* due to various environmental factors (e.g., air flushing and sodium nitrite) in the growth medium.

(*cntB*, the designation for the *C. botulinum* type B neurotoxin gene, was recommended by the ASM Publications Board Nomenclature Committee.)

MATERIALS AND METHODS

Bacterial strains and culture conditions. Three proteolytic strains (ATCC 7949, ATCC 17841, and Atlanta 3025) and two nonproteolytic strains (Eklund 2B and Eklund 17B) of *C. botulinum* type B were used (5). Overnight cultures of each *C. botulinum* strain were prepared in tryptone-peptone-yeast extract (TPY) broth and incubated anaerobically for 18 to 20 h at 37°C (proteolytic strains) or at 30°C (nonproteolytic strains) to obtain an optical density at 620 nm (OD₆₂₀) of 0.80 ± 0.05. The TPY broth contained tryptone (50 g/liter; Oxoid Ltd., Basingstoke, United Kingdom), proteose peptone (5 g/liter; Oxoid Ltd.), yeast extract (20 g/liter; Oxoid Ltd.), and sodium thioglycolate (1 g/liter; Merck, Darmstadt, Germany). Anaerobic conditions were created by boiling the medium for 10 min before sterilization (121°C for 15 min). After sterilization the medium was incubated in an anaerobic workstation (AW 800 TG; Electrotek, AddVise, Stockholm, Sweden) for 24 to 36 h before it was used. The atmosphere in the workstation contained nitrogen, carbon dioxide, and hydrogen (80:10:10). Flasks containing 285 ml of TPY-C broth (TPY broth supplemented with 0.4% glucose [BDH Laboratory Supplies, Poole, United Kingdom], 0.1% maltose [ICN Biochemicals Inc., Aurora, Ill.], 0.1% cellobiose [Sigma Chemical Co., St Louis, Mo.], and 0.1% soluble starch [Merck]) were each inoculated with 15 ml of an overnight single-strain culture and incubated under the conditions described above. Samples for extraction of total RNA were withdrawn after 0, 1, 2, 4, 5, 6, 7, 8, 9, 11, 12, 24, 28, 30, 32, 36, and 50 h of incubation. No samples were collected between 12 and 24 h because of the working hours of the laboratory. Before harvest, each cell suspension was quickly chilled on ice. At the same time, samples were removed to measure absorbance at 620 nm (model 330 spectrophotometer; G. K. Turner Associates, Palo Alto, Calif.), for ELISA analysis, and for analysis by the mouse bioassay. The specific growth rate of each strain was calculated by linear regression of the natural logarithm of the OD₆₂₀ versus time for the first OD₆₂₀ measurements that gave a linear relationship between the two variables. The growth experiment was performed independently twice with each strain.

RNA extraction. For RNA extraction a modified method for extraction of total RNA from *Bacillus* spp. with acidic phenol was used (30). The cells in a 10-ml suspension were harvested by centrifugation at 4°C and 16,000 × g for 10 min and resuspended in ice-cold TES buffer (50 mM Tris [ICN Biochemicals Inc.], 5 mM EDTA [Sigma Chemical Co.], 50 mM NaCl [Sigma Chemical Co.]; pH 7.5). Total RNA was recovered by simultaneous disruption and extraction in a solu-

tion containing acidic phenol (Aquaphenol; Saveen Biotech AB, Malmö, Sweden) and chloroform (Sigma Chemical Co.) (6:1). The cells were disrupted with 0.1-mm-diameter zirconia-silica beads (2.4 g) by using a bead miller (Mini-BeadBeater; BioSpec Products, Inc., Bartlesville, Okla.) at 5,000 rpm for 90 s. After precipitation at –70°C for 30 min, the RNA was collected by centrifugation at 4°C and 14,000 rpm for 20 min and resuspended in 200 µl of autoclaved Millipore-filtered water treated with diethyl pyrocarbonate (Sigma Chemical Co.). Before RT, contaminating DNA was degraded by treating 15 µl of each RNA sample with 15 U of RNase-free DNase (Promega Co., Madison, Wis.) and 1× reaction buffer. The reaction mixture was incubated at 37°C for 30 min, and the DNase was then inactivated by adding stop solution (20 mM EGTA, pH 8.0; Promega Co.) to the mixture and incubating it at 65°C for 10 min. Total RNA concentrations were determined with a RiboGreen RNA quantification kit (Molecular Probes, Inc., Leiden, The Netherlands) by measuring the fluorescence at 525 nm with a TD-700 fluorometer (Turner Designs, Sunnyvale, Calif.). The RNA was stored at –80°C before analysis.

Design of primers and fluorogenic probes. The primers and probes used in this study are listed in Table 1. Primers specific for the *rm* gene were designed from the nucleotide sequence of the 16S rRNA (accession number X68173) obtained from the GenBank sequence database (<http://www.ncbi.nlm.nih.gov>). The two fluorogenic probes, one specific for part of *cntB* and one specific for *rm*, contained a reporter dye (6-carboxyfluorescein) covalently attached at the 5' end and an internal quencher dye (Dark Quencher) attached to a deoxyuridine nucleotide. Extension from the 3' end was blocked by attachment of a 3' phosphate group. The fluorogenic probes were purified by high-performance liquid chromatography.

RT. First-strand cDNA was synthesized in two separate RT assays by using the reverse primers for *cntB* and *rm* (Table 1). cDNA synthesis was performed with a Gene Amp 9700 thermal cycler (Perkin-Elmer Cetus, Norwalk, Conn.). The total volume of the reaction mixture was 20 µl, and the mixture contained 0.5 µg of total RNA, each primer (Scandinavian Gene Synthesis AB, Köping, Sweden) at a concentration of 0.5 mM, each deoxynucleoside triphosphate (dATP, dTTP, dCTP, and dGTP; Amersham Pharmacia Biotech Inc., Piscataway, N.J.) at a concentration of 5 mM, 20 U of RNasin RNase inhibitor (Promega Co.), 5 mM dithiothreitol (Life Technologies Gaithersburg, Md.), 1× first-strand buffer, and 200 U of Superscript II RNase H⁻ reverse transcriptase (Life Technologies). Before RT enzymes were added, the reaction mixture was heated to 65°C for 5 min and then chilled on ice. After a brief centrifugation and addition of the RT enzymes, the reaction mixture was incubated at 42°C for 50 min, and the reaction was terminated by incubation at 70°C for 15 min. The cDNA solution was diluted 10-fold in autoclaved diethyl pyrocarbonate-treated water before PCR amplification.

qPCR. PCR amplification was carried out with a Lightcycler instrument (Roche Diagnostics GmbH, Mannheim, Germany). The total volume of the PCR master mixture was 20 µl. The amount of template solution (cDNA) added to each PCR mixture was 4 µl. The PCR mixture specific for *rm* consisted of 1× PCR buffer (Roche Diagnostics GmbH), each deoxynucleoside triphosphate (dATP, dTTP, dCTP, and dGTP; Amersham Pharmacia Biotech, Inc.) at a concentration of 0.2 mM, 5 mM MgCl₂ (Roche Diagnostics GmbH), each primer (Scandinavian Gene Synthesis AB) at a concentration of 0.7 µM, 0.7 µM fluorogenic probe (Scandinavian Gene Synthesis AB), and 0.05 U of *Tth* DNA polymerase (Roche Diagnostics GmbH). The PCR mixture specific for *cntB* consisted of 1× PCR buffer (Roche Diagnostics GmbH), each deoxynucleoside triphosphate (dATP, dTTP, dCTP, and dGTP; Amersham Pharmacia Biotech, Inc.) at a concentration of 0.2 mM, 4 mM MgCl₂ (Roche Diagnostics GmbH),

each primer (Scandinavian Gene Synthesis AB) at a concentration of 0.5 μM , 0.3 μM fluorogenic probe (Scandinavian Gene Synthesis AB), and 0.05 U of *Tth* DNA polymerase (Roche Diagnostics GmbH). The water used in the PCR assays was autoclaved Millipore-filtered water. Each sample was analyzed three times by the *cntB* and *rrn* real-time PCR assays. The following Lightcycler experimental run protocol was used for amplification: initial denaturation at 95°C for 1 min, 40 cycles of denaturation at 95°C for 0 s and combined annealing and extension at 58°C for 20 s with a single fluorescent measurement at the end of the extension, and finally cooling to 40°C. In each step the temperature transition rate was 20°C/s. The crossing point for each transcript was determined by using the second derivative maximum mathematical model in the Lightcycler software (version 3.5; Roche Molecular Biochemicals). The specificity of the PCRs was verified by ethidium bromide staining on 1.5% agarose gels. In order to check for amplification of any contaminating genomic DNA, a negative control was added to the PCR analysis. The negative control contained DNase-treated RNA, which was added to the PCR mixture without being reverse transcribed.

Total RNA was diluted 10-fold in the range from 5.2 μg to 5.2 pg, reverse transcribed, and amplified with the Lightcycler instrument in order to determine the amplification efficiency (E) and the log-linear range of amplification for each real-time PCR assay. The analysis was performed four times at each concentration of total RNA. The amplification efficiency in the exponential phase was calculated as follows: $E = 10^{(-1/s)} - 1$, where s is the slope of the log-linear range of amplification (17).

Relative quantification was based on the level of mRNA of *cntB* compared with the level of the reference gene, *rrn*, as described by Pfaffl (28). To quantify the transcript levels of *cntB* (i.e., the amounts of *cntB* mRNA that accumulated at time points), the same amount of total RNA (0.5 μg) from a culture at each growth phase was used in the RT procedure. The relative expression (RE) was calculated from the amplification efficiencies for each PCR assay and the crossing point deviation (ΔCp) of the unknown sample compared with a calibrator sample, as follows: $\text{RE} = (1 + E_{\text{cntB}})^{\Delta\text{Cp}_{\text{cntB}}(\text{calibrator-unknown sample})} / (1 + E_{\text{rrn}})^{\Delta\text{Cp}_{\text{rrn}}(\text{calibrator-unknown sample})}$.

ELISA. Samples collected from cultures of *C. botulinum* in TPY-C broth were used to quantify the production of both inactive and biologically active BoNT/B by an ELISA procedure with polyclonal antibodies specific for the type B BoNT from rabbits (Metabio, Inc., Madison, Wis.). *C. botulinum* ATCC 7949 and Eklund 2B were sampled at eight different times during the growth experiment. The remaining *C. botulinum* strains, ATCC 17841, Atlanta 3025, and Eklund 17B, were sampled at the end of growth (i.e., after 50 h). One milliliter of a cell culture was centrifuged at 4°C and 14,000 rpm for 10 min to separate the bacteria from the supernatant fluid. The supernatant was diluted in casein buffer so that the concentration was in the linear range of the BoNT/B standard concentrations (Metabio, Inc.). A 100- μl sample was placed in each well. The color change was measured with an ELISA multiscanner (Labsystem Multiscan Plus) at 492 nm by using the alkaline phosphatase system (ELISA amplification system; Life Technologies). BoNT/B standards were included on each plate at the following concentrations: 1, 0.9, 0.8, 0.7, 0.6, 0.5, and 0.4 ng/ml. Casein buffer was included as a negative control on each plate. Samples from both of the growth experiments for each strain were analyzed by the ELISA, and an average value and standard deviation were calculated.

Mouse bioassay. For *C. botulinum* ATCC 7949, samples obtained after 4, 6, 12, and 50 h of growth were analyzed to determine the effect of exposure to biologically active neurotoxin by the mouse bioassay (8, 22). For the *C. botulinum* Eklund 2B strain corresponding samples were collected after 4, 9, 12, and 50 h of growth. One milliliter of a cell culture was centrifuged at 4°C and 16,000 $\times g$ for 10 min to separate the bacteria from the supernatant fluid. The culture fluid (0.5 ml) was filter sterilized (pore size, 0.45 μm) before intraperitoneal injection into mice. In addition, the samples from the Eklund 2B strain culture were trypsinized in order to activate the BoNT/B by adding 1 part of 0.1% trypsin to 9 parts of supernatant fluid and incubating the preparations at 37°C for 30 min. Mice were observed for signs of botulism and death over a 5-day period. Toxicity was expressed as the number of hours until death.

Effect of air flushing of an early-stationary-phase cell culture. Two separate flasks containing 237.5 ml of TPY-C broth were inoculated with 12.5-ml samples of an overnight culture of exponentially growing *C. botulinum* ATCC 7949 (OD_{620} , 2.0) and incubated anaerobically at 37°C. Growth was monitored by determining the OD_{620} with a Hitachi UV-1500 spectrophotometer (Hitachi Instruments Inc.). When the cells reached the early stationary growth phase, each culture was exposed to filtered-sterilized air (pore size, 0.2 μm) by flushing the air through the growth medium with a gas distributor and a magnetic stirrer for 10 min at a constant rate of 0.25 liter/min. Samples used for extraction of total RNA were collected at 0, 80, 160, and 240 min after the end of the exposure. Samples were withdrawn at the same times for viable counting on blood agar

plates containing blood agar base (37 g/liter; Lab M, Bury, United Kingdom) and citrate-treated horse blood (4% [vol/vol]; SVA, Uppsala, Sweden).

Effect of adding sodium nitrite to the growth medium. Flasks containing 237.5 ml of TPY-C broth were inoculated with 12.5 ml of an overnight culture of exponentially growing *C. botulinum* Eklund 2B (OD_{620} , 1.0) and incubated anaerobically at 30°C. Sodium nitrite was added to the flasks before sterilization at the following concentrations: 0, 15, 30, and 45 ppm. Growth was monitored by determining the OD_{620} with the Hitachi UV-1500 spectrophotometer. Samples used for extraction of total RNA were withdrawn three times during growth, in the exponential phase, in the late exponential phase, and in the late stationary phase. The relative expression of *cntB* was calculated as described above for each growth phase and each sodium nitrite concentration.

RESULTS

Development of qRT-PCR. Two fluorogenic probes were designed; one was specific for *cntB*, and the other was specific for *rrn* (Table 1). Agarose gel electrophoresis analysis showed that the qRT-PCR for *cntB* amplified a PCR product of the predicted size, 0.22 kb, and the qRT-PCR for *rrn* resulted in a 0.21-kb PCR product, as expected. In order to determine the amount of total RNA that should be added during the RT step, the linear ranges of amplification for the two qRT-PCR assays were established. The linear range of amplification and the amplification efficiency for each qRT-PCR assay were determined by dilution of the total RNA over 8 log units before the RNA was added to the RT mixture. The *cntB* assay had a linear range of amplification of between 5.2 μg and 5.2 ng of added total RNA, and the amplification efficiency was determined to be 1.11. The *rrn* assay had a linear range of amplification of between 0.52 μg and 5.2 pg of added total RNA, and the amplification efficiency was 1.14.

To check the consistency of the DNase treatment and to make sure that it was the mRNA, not any contaminating genomic DNA that was coextracted with the total RNA, that was amplified, DNase-treated RNA was amplified by real-time PCR assays throughout the experiments. None of the controls gave any fluorescent signal in the real-time PCR assays, indicating that the DNase treatment removed all genomic DNA (data not shown).

Relative expression of *cntB*. The specific growth rate, the level of *cntB* mRNA, the extracellular BoNT/B concentration, and the toxicity of the biologically active neurotoxin formed were determined from two independent growth experiments for each of the five *C. botulinum* strains (Fig. 1 and 2). The specific growth rates for the strains were as follows: *C. botulinum* ATCC 7949, $0.81 \pm 0.08 \text{ h}^{-1}$; *C. botulinum* ATCC 17841, $0.96 \pm 0.03 \text{ h}^{-1}$; *C. botulinum* Atlanta 3025, $1.06 \pm 0.15 \text{ h}^{-1}$; *C. botulinum* Eklund 2B, $0.89 \pm 0.08 \text{ h}^{-1}$; and *C. botulinum* Eklund 17B, $0.99 \pm 0.33 \text{ h}^{-1}$.

Total RNA was extracted from cell cultures during the incubation period for each of the strains. The same amount of total RNA (0.5 μg) from each cell culture was used for relative quantification of the transcript level of the mRNA of the *cntB* gene. In order to correct for sample-to-sample variation, the *cntB* transcript was normalized to the reference gene, *rrn*. In these experiments, the *rrn* gene was consistently expressed after 4 h of growth and was therefore used as the calibrator sample (relative expression, 1.00) to which all the other samples were compared in the calculations of relative expression.

Overall, *cntB* mRNA was detected at all stages of growth in the five *C. botulinum* strains investigated. The highest levels of

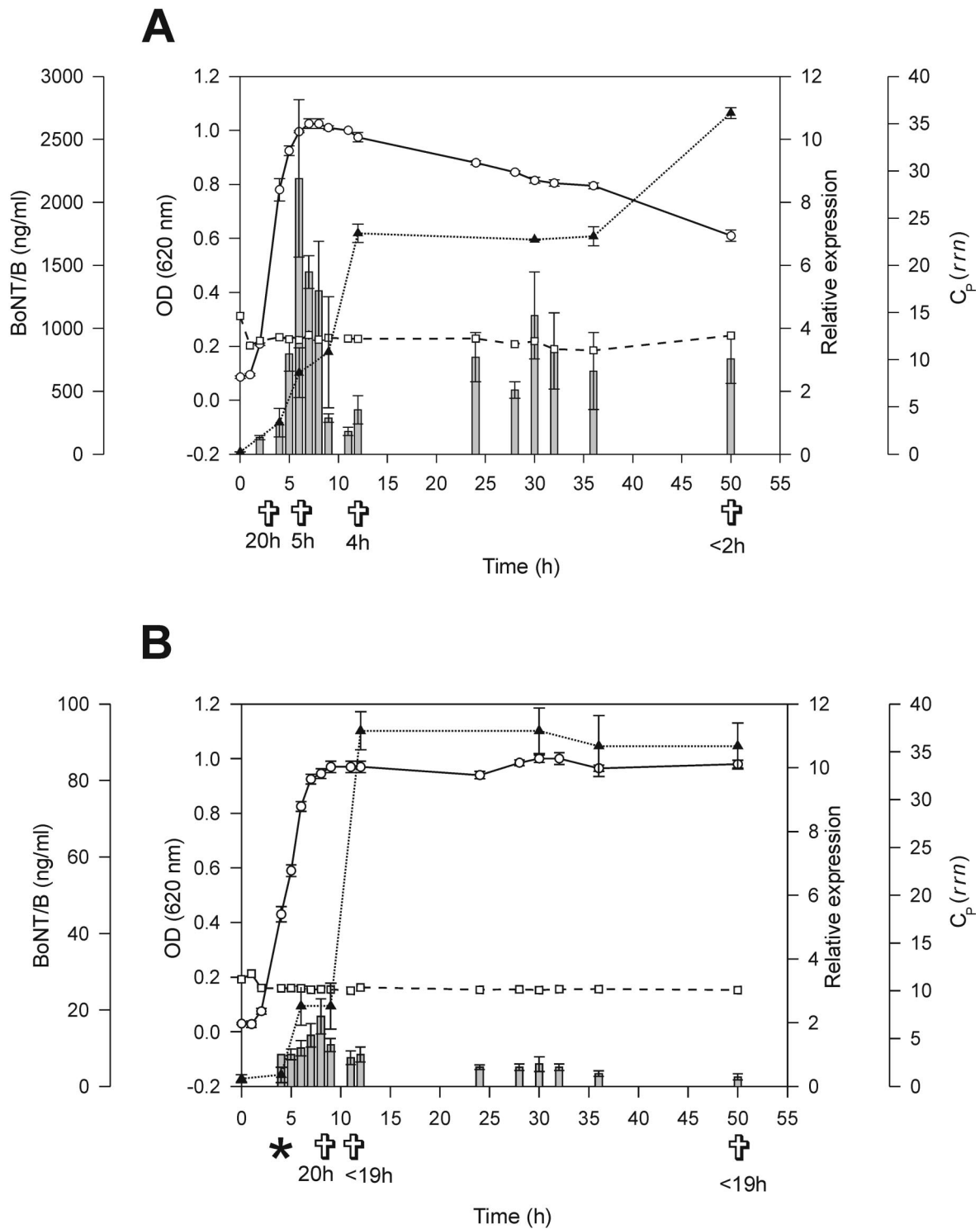


FIG. 1. Correlation of relative expression of the *cntB* gene and extracellular BoNT/B formation during the growth cycles of the proteolytic strain *C. botulinum* ATCC 7949 (A) and the nonproteolytic strain *C. botulinum* Eklund 2B (B). ○, growth curve as determined by measurement of OD₆₂₀; □, crossing point (C_p) values of the reference gene, *rrn*; bars, relative expression of the *cntB* gene; ▲, extracellular BoNT/B concentration as determined by the specific ELISA. A dagger indicates toxicity expressed as the number of hours until death was observed in the mouse bioassay; an asterisk indicates that no BoNT/B was detected by the mouse bioassay. The values are averages and standard deviations based on two independent growth experiments. The standard deviations for *rrn* are not larger than the symbols.

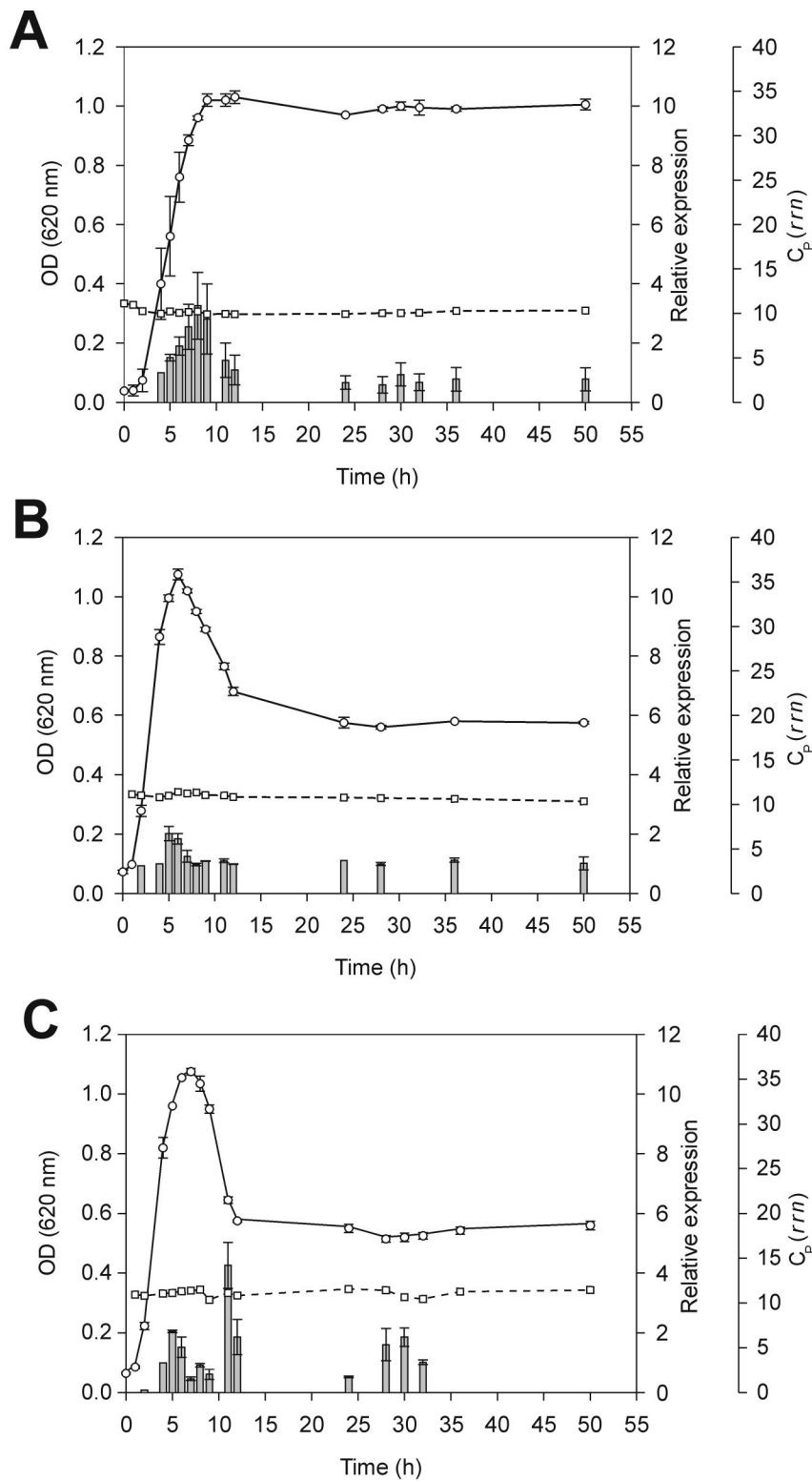


FIG. 2. Relative expression of *cntB* during the growth cycles of the nonproteolytic strain *C. botulinum* Eklund 17B (A), the proteolytic strain *C. botulinum* Atlanta 3025 (B), and the proteolytic strain *C. botulinum* ATCC 17841 (C). \circ , growth curve as determined by measurement of OD_{620} ; \square , crossing point (C_p) values of the reference gene, *rrn*; bars, relative expression of *cntB*. The values are averages and standard deviations based on two independent growth experiments. The standard deviations for *rrn* are not larger than the symbols.

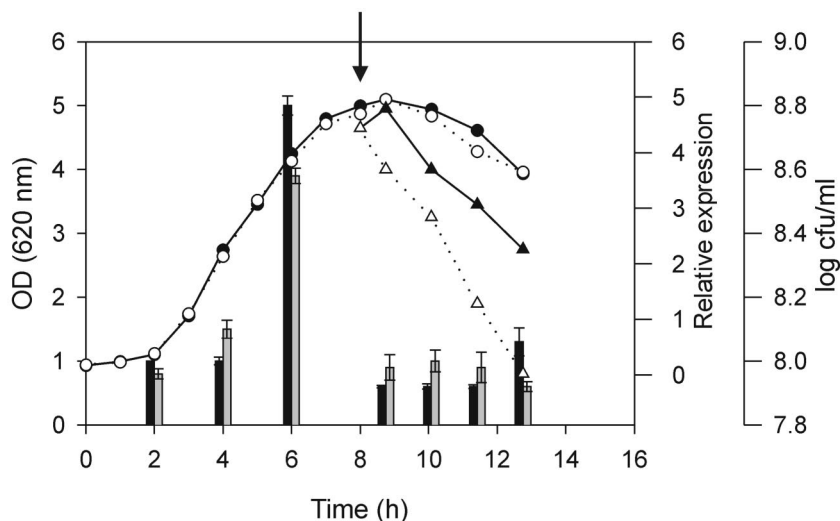


FIG. 3. Effect of flushing sterilized air through the growth medium for 10 min in an early-stationary-phase cell culture of *C. botulinum* ATCC 7949 on the relative *cntB* expression in relation to growth. The duration of the exposure to air is indicated by an arrow. ●, growth curve of the untreated cell culture as determined by measurement of OD₆₂₀; ○, growth curve of the air-treated cell culture as determined by measurement of OD₆₂₀; solid bars, relative expression of *cntB* in the untreated cell culture; shaded bars, relative expression of *cntB* in the air-treated cell culture; ▲, viable counts (log CFU per milliliter) in the untreated cell culture; △, viable counts (log CFU per milliliter) in the air-treated cell culture.

cntB mRNA were observed in *C. botulinum* ATCC 7949 (Fig. 1A). During growth of this strain the level of *cntB* mRNA was threefold greater in the late exponential phase than in the exponential phase. When the cells were entering the early stationary phase, the level of *cntB* mRNA was even higher, and it was approximately 10-fold greater than the level in the exponential phase. In the stationary phase, the level of *cntB* mRNA was comparable to the level in the exponential phase. In addition, the mRNA transcript level in the death phase was comparable to that in the late exponential phase except after 30 h, when the *cntB* mRNA level was higher. The presence of extracellular BoNT/B, as determined by the ELISA, was observed as soon as growth occurred, and the concentration increased to a maximum value of about 1,750 ng/ml in the stationary phase. The highest concentration of BoNT/B measured, more than 2,500 ng/ml, was observed after 50 h of growth. At this point, when the mouse bioassay was used, the effect of exposure to the active neurotoxin was observed within 2 h after injection (Fig. 1A). In addition, at the highest level of *cntB* mRNA (in the late exponential phase), the toxic effect of the neurotoxin was detected 5 h after injection. In the early exponential phase the effect of exposure to the toxin was detected only after 20 h. In general, the relative levels of the neurotoxin mRNA of the other four strains were lower than those of *C. botulinum* ATCC 7949. However, the pattern was the same as the pattern observed for ATCC 7949; there was an increase in expression when the bacteria entered the stationary phase. For *C. botulinum* Eklund 2B the *cntB* mRNA level in the early stationary phase was twofold greater than the level in the exponential phase. During the stationary phase the relative mRNA concentration continued to decline to levels lower than those in the exponential phase. The extracellular BoNT/B detected by the ELISA also showed a pattern similar to that observed with ATCC 7949; the level increased until the bacteria reached the stationary phase. However, the BoNT/B levels were between 18- and 25-fold lower in Eklund 2B than in

ATCC 7949, and the highest toxin concentration was around 100 ng/ml in the stationary phase. The corresponding toxin concentrations detected at the end of growth (50 h) for the remaining strains were as follows: *C. botulinum* Eklund 17B, 230 ng/ml; *C. botulinum* Atlanta 3025, 1,040 ng/ml; and *C. botulinum* ATCC 17841, 730 ng/ml. With Eklund 2B the effect of exposure to BoNT/B was observed 19 h after injection in the stationary phase and after 20 h at a toxin concentration around 20 ng/ml. At the lowest BoNT/B concentration (2 ng/ml) no effect of exposure to toxin was detected by the mouse bioassay.

Effects of air and sodium nitrite on growth and *cntB* expression. As *C. botulinum* is an obligately anaerobic microorganism, exposure to molecular oxygen may be toxic to the bacteria, resulting in a reduction in bacterial growth or even death and a subsequent decrease in *cnt* gene expression. In this study, the effect of flushing air through the growth medium of an early-stationary-phase cell culture of *C. botulinum* ATCC 7949 was investigated by examining growth and the level of *cntB* mRNA. The cell concentration was 8.7 log CFU/ml before flushing was initiated. The OD₆₂₀ values of the air-flushed cell culture were similar to those of the anaerobically incubated cell culture (Fig. 3). However, the reduction in viable counts after 4 h was 0.77 log CFU/ml for the air-flushed cell culture, compared to 0.37 log CFU/ml for the untreated cell culture. No significant difference in the *cntB* mRNA level in the stationary phase was detected in the two cell cultures. Instead, the level of *cntB* mRNA in the air-treated cell culture was maintained (Fig. 3).

Sodium nitrite (NaNO₂) is a well-known inhibitor of bacterial growth in *C. botulinum*. In this study, the effects of sodium nitrite on growth and the mRNA transcript level of *cntB* were determined for *C. botulinum* Eklund 2B when different concentrations were added to the growth medium before sterilization. Samples were removed three times during growth (in the exponential growth phase, in the late exponential growth phase, and in the stationary growth phase) and used for *cntB* mRNA analysis. No significant differences in growth or the

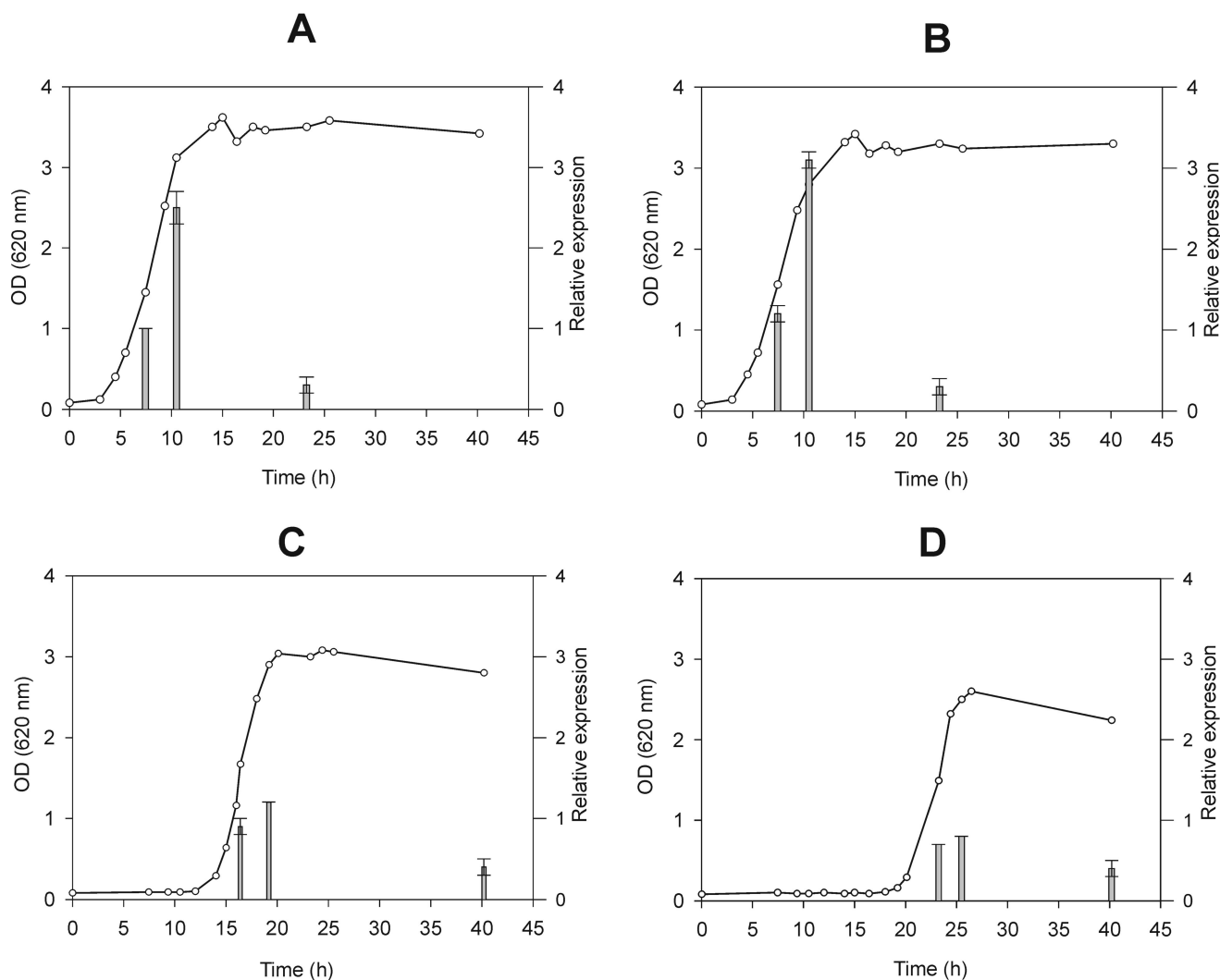


FIG. 4. Effects of different concentrations of sodium nitrite (NaNO_2) on the relative *cntB* expression in relation to the growth of *C. botulinum* Eklund 2B. NaNO_2 was added to TPY-C medium before sterilization. (A) No NaNO_2 ; (B) 15 ppm of NaNO_2 ; (C) 30 ppm of NaNO_2 ; (D) 45 ppm of NaNO_2 . \circ , growth curve as determined by measurement of OD_{620} ; bars, relative expression of *cntB*.

cntB mRNA level were observed at NaNO_2 concentrations between 0 and 15 ppm (Fig. 4A and B). The specific growth rate was 0.83 h^{-1} when no NaNO_2 was added, compared with 0.82 h^{-1} when 15 ppm of NaNO_2 was added. However, at higher concentrations of NaNO_2 a slight growth effect was observed (Fig. 4C and D). When higher concentrations of NaNO_2 were used, the duration of the lag phase was longer and the growth rate decreased (0.78 h^{-1} at 30 ppm and 0.70 h^{-1} at 45 ppm). In addition, there was a decrease in the relative *cntB* mRNA level, especially in the late exponential phase in the presence of 30 and 45 ppm of NaNO_2 . In the presence of 45 ppm of NaNO_2 the reduction in the *cntB* mRNA level in all growth phases was significant.

DISCUSSION

RT followed by real-time PCR is a powerful tool for analysis of mRNA expression due to its great sensitivity and ability to quantify even small changes in gene expression (10, 14, 33). In this paper, we report development of a novel qRT-PCR

method for quantitative analysis of *cntB* mRNA in *C. botulinum* type B. The relative quantity of the *cntB* transcript was determined by comparison to the quantity of a reference gene, *rm*, and the data were based on corrections for different amplification efficiencies in the two real-time PCR assays (28). The relative expression of *rm* was constant throughout the mid-exponential, late-exponential, and late-stationary growth phases (Fig. 1). A constant amount of total RNA from each cell culture had to be added to the RT reaction mixture for correct quantification. In the present study, this amount (0.5 μg) was derived from the linear range of amplification for the two PCR assays.

Only limited data are available regarding the kinetics of growth of *C. botulinum* and expression of the BoNT gene. In this work, the five type B *C. botulinum* strains investigated with the qRT-PCR method clearly differed in the levels of *cntB* mRNA during their growth cycles (Fig. 1 and 2). In four of the strains the maximum level of *cntB* mRNA was observed in the early stationary phase, whereas in the fifth strain, *C. botulinum*

ATCC 17841, the maximum level was seen later in the stationary phase. Similar results showing that there was maximal toxin expression as the bacteria entered the stationary growth phase have been obtained previously for *C. botulinum* type E (21) and *Clostridium difficile* (9, 16). The highest levels of neurotoxin expression in all growth phases were exhibited by the proteolytic strain *C. botulinum* ATCC 7949. In the early stationary phase the level of *cntB* mRNA was approximately 10-fold higher than levels in the early exponential phase. The corresponding levels in the other *C. botulinum* strains, Eklund 2B, Eklund 17B, and Atlanta 3025, were four- to fivefold lower.

The relative levels of *cntB* mRNA definitely reflected the BoNT/B concentration and the toxicity when data for *C. botulinum* ATCC 7949 and *C. botulinum* Eklund 2B were analyzed. When detecting the production of extracellular BoNT/B with specific antibodies by the ELISA method, we observed that *C. botulinum* ATCC 7949 produced the highest concentrations of the neurotoxin. During the exponential phase there was an increase in the BoNT/B level, and the first maximum was in the early stationary growth phase. The time difference between the buildup of *cntB* mRNA and the extracellular buildup of BoNT/B was approximately 6 h. This was probably because formation of the extracellular toxin was measured in the supernatant fluid and not intracellularly. Call et al. (3), who examined BoNT/A synthesis, translocation, and export in a *C. botulinum* type A strain by using antibody-coated colloidal gold probes and electron microscopy, also observed an increase in the level of the extracellular neurotoxin in the early stationary phase. The level of the extracellular BoNT/B in *C. botulinum* Eklund 2B also increased during exponential growth and reached a maximum in the early stationary growth phase. During the remainder of the stationary phase the neurotoxin concentration was constant. The same behavior was observed with *C. botulinum* ATCC 7949 until the bacteria reached the death phase, when there was a second increase in the BoNT/B concentration, from around 1,750 ng/ml to over 2,500 ng/ml. There is no evidence in the literature that lysis is a prerequisite for neurotoxin release from the cell, which might provide an explanation for the observed increase in this study (3, 31). Furthermore, Call et al. (3) observed that when a *C. botulinum* type A cell culture reached the death phase, cells containing endospores were detected; accumulation of BoNT/A was associated with these spores, and BoNT/A was found in the mother cells. However, in the present study no endospores were observed in the cells in the death phase (after 50 h of growth). On the other hand, there was a decrease in the viable count from 8.3 log CFU/ml in late stationary phase to 7.4 log CFU/ml in the death phase, which could indicate that there was autolysis of cells and that the neurotoxin was released into the surrounding medium.

A higher titer of biologically active BoNT/B toxin was also observed in the cell culture in the death phase when it was analyzed by the mouse bioassay; the effect of exposure to the neurotoxin was detected within 2 h, compared with an early-stationary-phase cell culture of *C. botulinum* ATCC 7949, in which the effect of BoNT/B was detected within 4 h. In strain Eklund 2B a lower *cntB* mRNA level corresponded to both lower BoNT/B concentrations and lower toxicity. At a very low

neurotoxin concentration, 2 ng/ml, no effect of exposure to BoNT/B was detected by the mouse bioassay.

Very little is known about the nutritional and environmental factors that regulate neurotoxin production in *C. botulinum* in growth media and in different foods. Previous studies have shown that the important factors include nitrogenous nutrients, such as arginine and tryptophan, which have both been shown to repress BoNT production, and casein, which stimulates BoNT formation (18, 24). In the present study, the effects of air and sodium nitrite on the level of *cntB* mRNA were determined.

C. botulinum is an obligately anaerobic bacterium, and molecular oxygen may be toxic due to the bacterium's lack of the enzymes catalase and superoxide dismutase (1). When flushing an early-stationary-phase cell culture with sterile air, we observed a toxic effect on bacterial growth, resulting in a rapid decrease in the viable count to only 17% of the initial cell number, compared with a 42% decrease in the untreated cell culture. However, in spite of the reduction in the viable count, no significant effect on the relative level of *cntB* mRNA was observed after exposure to air. Instead, the level of *cntB* mRNA in the air-treated cell culture was maintained.

Sodium nitrite plays a major role in the botulinal safety of cured meat products because it delays both outgrowth from *C. botulinum* spores and vegetative growth of the bacteria (32). This compound has been used as a preservative in the food industry for many years, but its use is often controversial due to reports showing that when it is added to meats, it acts as a precursor of carcinogenic nitrosamines. Improved information concerning the effect of sodium nitrite on *cnt* gene expression should provide important clues to its effectiveness. Furthermore, the inhibitory effect of heated sodium nitrite in a microbial growth medium is not identical to the effect in cured meats, which is often referred to as the Perigo effect (26, 27). In the present study, the effect of sodium nitrite that was heat treated in the growth medium on growth and on *cntB* mRNA was investigated. At sodium nitrite concentrations higher than 15 ppm an inhibitory effect on both the lag phase and growth was detected. The bacteria needed a longer time to adjust to the media with higher sodium nitrite concentrations before they started to grow exponentially, and, in addition, the growth rates were lower. Furthermore, there was a reduction in the level of *cntB* mRNA at higher concentrations. This was most obvious in the late exponential growth phase, but at the highest sodium nitrite concentration, 45 ppm, a decline in the level of *cntB* mRNA was observed in all of the growth phases.

Moreover, in bacteriological gene expression studies the levels of expression of various housekeeping genes may change when bacteria are grown under different conditions. It is known that when bacterial growth slows, the content of ribosomes may decrease along with the rate of rRNA synthesis (12). Therefore, when *C. botulinum* grows in the presence of nitrite, conditions in which cell growth has a long lag phase and then proceeds more slowly than growth of the control cells, the ratio of *cntB* to *rrn* may not be comparable to the ratio in fast-growing cells. If the growth conditions reduce *rrn* transcription, the *cntB/rrn* ratio may be even higher in such cells than in untreated cells. The fact that the *cntB/rrn* ratio is lower in nitrite-treated cells suggests that the *cntB* mRNA level may be even lower than it appears to be. However, in our experi-

ments the *rn* gene was constantly expressed throughout the mid-exponential, late-exponential, and late-stationary growth phases when the cells were exposed to nitrite or air (data not shown).

In conclusion, the qRT-PCR method described here is a valuable tool for monitoring *cnt* gene expression in *C. botulinum* and for increasing our knowledge about nutritional and environmental regulation of the BoNT gene. The *cntB* mRNA levels were successfully quantified in microbial growth media, and the effects of air and sodium nitrite on expression were determined. This method could be used to study neurotoxin expression and factors that influence BoNT formation in foods. One challenge is to isolate high-quality prokaryotic RNA from different food matrices. In addition, the qRT-PCR method could be used as an alternative or as a complement to the mouse bioassay for medical diagnosis of botulism.

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