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## Quantitative Interaction Effects of Carbon Dioxide, Sodium Chloride, and Sodium Nitrite on Neurotoxin Gene Expression in Nonproteolytic *Clostridium botulinum* Type B

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The effects of carbon dioxide, sodium chloride, and sodium nitrite on type B botulinum neurotoxin (BoNT/B) gene (*cntB*) expression in nonproteolytic *Clostridium botulinum* were investigated in a tryptone-peptone-yeast extract (TPY) medium. Various concentrations of these selected food preservatives were studied by using a complete factorial design in order to quantitatively study interaction effects, as well as main effects, on the following responses: lag phase duration (LPD), growth rate, relative *cntB* expression, and extracellular BoNT/B production. Multiple linear regression was used to set up six statistical models to quantify and predict these responses. All combinations of NaCl and NaNO<sub>2</sub> in the growth medium resulted in a prolonged lag phase duration and in a reduction in the specific growth rate. In contrast, the relative BoNT/B gene expression was unchanged, as determined by the *cntB*-specific quantitative reverse transcription-PCR method. This was confirmed when we measured the extracellular BoNT/B concentration by an enzyme-linked immunosorbent assay. CO<sub>2</sub> was found to have a major effect on gene expression when the *cntB* mRNA levels were monitored in the mid-exponential, late exponential, and late stationary growth phases. The expression of *cntB* relative to the expression of the 16S rRNA gene was stimulated by an elevated CO<sub>2</sub> concentration; the *cntB* mRNA level was fivefold greater in a 70% CO<sub>2</sub> atmosphere than in a 10% CO<sub>2</sub> atmosphere. These findings were also confirmed when we analyzed the extracellular BoNT/B concentration; we found that the concentrations were 27 ng · ml<sup>-1</sup> · unit of optical density<sup>-1</sup> in the 10% CO<sub>2</sub> atmosphere and 126 ng · ml<sup>-1</sup> · unit of optical density<sup>-1</sup> in the 70% CO<sub>2</sub> atmosphere.

In recent years, nonproteolytic strains of *Clostridium botulinum* have become a subject for concern due to the development of novel food products having alternative formulations and produced by different processing techniques (e.g., refrigerated processed foods with extended durability) (26). These food products are usually pasteurized at temperatures that destroy vegetative bacteria but do not kill spores, are packed under an anaerobic atmosphere, and are then stored at refrigerated temperatures. These characteristics are favorable for spore germination, outgrowth, and subsequent neurotoxin formation in the food if nonproteolytic *C. botulinum* is present, as this bacterium has been reported to multiply and produce type B, E, or F botulinum neurotoxins (BoNTs) at temperatures as low as 3.0°C (11). To ensure food safety in these refrigerated food products, additional control measures must be employed; these measures include adjustment of the pH and addition of sodium chloride and other food preservatives to prevent growth and formation of neurotoxin by *C. botulinum*.

In previous studies on *C. botulinum* workers have focused mainly on the germination and outgrowth of spores in both microbiological growth medium and different foods (10, 18, 22, 27). However, little is known about the environmental factors

that regulate BoNT gene (*cnt*) expression in *C. botulinum*. Nutritional conditions affect BoNT formation in *C. botulinum*, and it has been reported that nitrogen sources, such as tryptophan and arginine, repress BoNT production (16, 25), whereas casein induces synthesis of the toxin (6, 16). The *botR* gene encoding a 22-kDa protein has been localized in the *cnt* gene cluster of all serotypes except serotype E (4, 12, 13, 29). Furthermore, the protein encoded by this gene, BotR, has been recognized as a positive regulator of the *cntB* gene in *C. botulinum* type A (21). BotR is related to other regulatory proteins, such as TetR of *Clostridium tetani* (20) and TxeR of *Clostridium difficile* (23).

To better understand regulation of *cnt* gene expression in *C. botulinum*, it is necessary to identify the environmental factors in different foods that induce or repress neurotoxin production. A quantitative reverse transcription-PCR (qRT-PCR) method was recently developed for relative quantification of type B *cnt* (*cntB*) expression (19). The results obtained showed that the maximum *cntB* mRNA accumulation occurred as the bacteria entered the early stationary growth phase. Furthermore, it was found that exposure to air had no effect on *cntB* expression, whereas a sodium nitrite concentration of 45 ppm suppressed *cntB* expression.

In the present study, the effects of selected food preservatives on *cntB* expression in a nonproteolytic *C. botulinum* Ek-lund type B strain were investigated by using the *cntB*-specific qRT-PCR method (19). Various concentrations of carbon di-

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oxide, sodium chloride, and sodium nitrite were studied in a factorial-design experiment in order to quantitatively study interaction effects, as well as main effects, on the following responses: lag phase duration (LPD), growth rate, relative *cntB* expression, and neurotoxin production. Fifty-four independent growth experiments were performed with a tryptone-peptone-yeast extract (TPY) medium, and the *cntB* expression was measured three times during the growth cycle: in the mid-exponential, late exponential, and late stationary growth phases. BoNT/B production was quantified by an enzyme-linked immunosorbent assay (ELISA). Multiple linear regression was used to set up six models to quantify and predict the responses.

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

#### MATERIALS AND METHODS

**Bacterial strain and culture conditions.** The nonproteolytic strain *C. botulinum* Eklund 2B was used throughout this study and was maintained as described previously (5). Overnight cultures were prepared in TPY broth (17) and incubated anaerobically for 18 to 20 h at 30°C until the optical density at 620 nm ( $OD_{620}$ ) was  $1.2 \pm 0.1$ . 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 for 24 to 36 h before use in an anaerobic workstation (AW 800 TG; Electrotek, AddVise, Stockholm, Sweden). The atmosphere in the workstation contained nitrogen, carbon dioxide, and hydrogen (80:10:10).

A factorial design was used to study the effects of carbon dioxide ( $CO_2$ ), sodium chloride (NaCl), and sodium nitrite ( $NaNO_2$ ) on the LPD, the specific growth rate, the relative *cntB* expression, and the extracellular BoNT/B concentration. Various amounts of NaCl (final concentrations, 0, 1.25, and 2.5% [wt/vol]; Merck) and  $NaNO_2$  (final concentrations, 0, 37.5, and 75 ppm; Sigma Chemical Co., St Louis, Mo.) were added before sterilization to TPY-C basal medium, which was TPY medium supplemented with 0.4% (wt/vol) glucose (BDH Laboratory Supplies, Poole, United Kingdom), 0.1% (wt/vol) maltose (ICN Biochemicals Inc., Aurora, Ill.), 0.1% (wt/vol) cellobiose (Sigma Chemical Co.), and 0.1% (wt/vol) soluble starch (Merck) (24). Three modified atmospheres with different levels of  $CO_2$  were tested; these atmospheres contained 10, 35, and 70% (vol/vol)  $CO_2$ . The concentration of  $H_2$  was maintained at 10% (vol/vol) in all three gas mixtures. Nine flasks containing TPY-C medium and the various concentrations of NaCl and  $NaNO_2$  were flushed with each of the three gas mixtures. Before flushing of the medium, the gas was passed through a sterile filter with a pore size of 0.2  $\mu$ m. The gas was flushed through the medium for 10 min at a constant rate of 0.25 liter/min by using a gas distributor and a magnetic stirrer. After flushing, the medium was equilibrated for 24 to 36 h at 30°C in an anaerobic workstation with the same atmosphere. The remaining oxygen in the medium was measured with an oxygen sensor (Inpro 6000; Mettler Toledo, Steinbach, Germany) after equilibration in the anaerobic workstation to ensure that it was anaerobic. The initial pH was 7.0 for all flasks, indicating that it was not influenced by the  $CO_2$ .

The factorial design resulted in 27 different medium combinations (Table 1). Each flask containing 237.5 ml of TPY-C broth was inoculated with 12.5 ml of an overnight culture of *C. botulinum* Eklund 2B and incubated under the conditions described above. Growth was monitored by measuring the  $OD_{620}$  with an Hitachi UV-1500 spectrophotometer (Hitachi Instruments Inc.). The duration of each growth experiment was 10 days. The pH was measured each time that samples were withdrawn for measurement of  $OD_{620}$ . Samples used for extraction of total RNA were withdrawn three times during growth: in the middle of the exponential growth phase, in the early stationary growth phase, and in the late stationary growth phase. The growth experiment was performed twice with each of the 27 medium combinations. The specific growth rate was calculated by linear regression of the natural logarithm of the  $OD_{620}$  versus time (in hours) for the first  $OD_{620}$  measurements that gave a linear relationship between the two variables. The LPD and the maximum  $OD_{620}$  values were determined by using the Baranyi

TABLE 1. Experimental design and results obtained when we studied the interaction effects of NaCl,  $NaNO_2$ , and  $CO_2$  on the LPD, specific growth rate, and the maximum optical density

Expt	Exptl design			LPD (h) <sup>a</sup>	Specific growth rate (h <sup>-1</sup> ) <sup>a</sup>	OD <sub>620</sub> <sup>a</sup>
	NaCl concn (% wt/vol)	$NaNO_2$ concn (ppm)	$CO_2$ concn (%)			
1	0	0	10	3.0, 3.5	1.14, 1.12	4.4, 4.4
2	0	37.5	10	19.5, 19.5	0.73, 0.69	2.7, 2.7
3	0	75	10	24.5, 24.5	0.61, 0.61	2.9, 2.9
4	1.25	0	10	4.5, 4.5	0.81, 0.79	4.5, 3.8
5	1.25	37.5	10	17.0, 17.0	0.75, 0.69	2.7, 2.6
6	1.25	75	10	24.4, 24.6	0.38, 0.36	2.9, 2.9
7	2.5	0	10	23.0, 28.0	0.42, 0.53	2.8, 1.6
8	2.5	37.5	10	NG <sup>b</sup>	NG	NG
9	2.5	75	10	NG	NG	NG
10	0	0	35	3.5, 4.5	0.87, 0.80	4.5, 4.3
11	0	37.5	35	16.0, 17.0	0.87, 0.85	3.1, 3.1
12	0	75	35	26.5, 18.0	0.75, 0.56	3.5, 3.5
13	1.25	0	35	5.5 <sup>c</sup>	0.67 <sup>c</sup>	3.9 <sup>c</sup>
14	1.25	37.5	35	15.5, 18.0	0.68, 0.69	3.0, 2.9
15	1.25	75	35	18.0, 18.0	0.62, 0.58	2.9, 2.7
16	2.5	0	35	26.5 <sup>c</sup>	0.41 <sup>c</sup>	1.9 <sup>c</sup>
17	2.5	37.5	35	NG	NG	NG
18	2.5	75	35	NG	NG	NG
19	0	0	70	6.5 <sup>c</sup>	0.66 <sup>c</sup>	4.0 <sup>c</sup>
20	0	37.5	70	37.0 <sup>c</sup>	0.48 <sup>c</sup>	3.1 <sup>c</sup>
21	0	75	70	69.0 <sup>c</sup>	0.49 <sup>c</sup>	2.4 <sup>c</sup>
22	1.25	0	70	6.5, 5.0	0.66, 0.66	2.7, 2.5
23	1.25	37.5	70	32.0, 32.0	0.39, 0.43	2.6, 2.5
24	1.25	75	70	62.5, 63.5	0.29, 0.42	2.3, 2.5
25	2.5	0	70	32.0, 35.5	0.09, 0.12	2.6, 2.4
26	2.5	37.5	70	NG	NG	NG
27	2.5	75	70	NG	NG	NG

<sup>a</sup> Data obtained from two experiments unless indicated otherwise.

<sup>b</sup> NG, no growth.

<sup>c</sup> Data obtained from a single experiment.

growth model (1) and R software, version 1.6.1 (Rfit; <http://umr5558-sud-str3.univ-lyon1.fr/Rfit/>) (3).

**Analysis of *cntB* expression and BoNT/B production.** For extraction of total RNA a modified method for extraction of total RNA from *Bacillus* spp. with acidic phenol was used (30). Ten milliliters of the suspension was removed from each flask after shaking and chilled on ice, and the cells were harvested by centrifugation at 4°C and  $10,400 \times g$  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 the method described in the accompanying paper (19). Before reverse transcription, contaminating DNA was degraded by treatment with DNase (Promega Co., Madison, Wis.). Total RNA concentrations were determined with a RiboGreen RNA quantification kit (Molecular Probes, Inc., Leiden, The Netherlands). The concentrations were determined 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.

The *cntB*-specific qRT-PCR method used in this study is described in the accompanying paper (19). To quantify the transcript levels of *cntB*, the same amount of total RNA (0.5  $\mu$ g) from each growth phase culture was used in the reverse transcription reaction. PCR amplification was carried out with a Lightcycler instrument (Roche Diagnostics GmbH, Mannheim, Germany). 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). Specific primers were used in two separate PCR assays; one of the primers was specific for *cntB*, and the other was specific for *rrn* encoding the 16S rRNA. The two fluorogenic probes, one for *cntB* and one for *rrn*, contained a reporter dye (6-carboxyfluorescein) covalently attached at the 5' end and an internal quencher dye (Dark Quencher) attached to a deoxyuridine nucleotide (19). 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.

Relative quantification was based on the mRNA level of *cntB* compared with

TABLE 2. Effects on LPD and growth rate of *C. botulinum* Eklund 2B<sup>a</sup>

Dependent variable	Coefficient	Effect	95% confidence interval
LPD	$b_0^i$	5.6	1.4, 9.7
	$b_{13}^i$	50	40, 58
	$b_{22}^i$	21	12, 29
Specific growth rate	$b_0^{ii}$	1.0	0.9, 1.1
	$b_3^{ii}$	-0.4	-0.6, -0.3
	$b_{12}^{ii}$	0.3	0.1, 0.5
	$b_{22}^{ii}$	-0.5	-0.6, -0.4
	$b_{11}^{ii}$	-0.4	-0.5, -0.3

<sup>a</sup> The effect of CO<sub>2</sub> is indicated by 1, the effect of NaCl is indicated by 2, and the effect of NaNO<sub>2</sub> is indicated by 3, so that  $b_3$  is the effect of [NaNO<sub>2</sub>],  $b_{11}$  is the effect of [CO<sub>2</sub>]<sup>2</sup>,  $b_{22}$  is the effect of [NaCl]<sup>2</sup>,  $b_{13}$  is the effect of [CO<sub>2</sub>] · [NaNO<sub>2</sub>], and  $b_{12}$  is the effect of [CO<sub>2</sub>] · [NaCl]. The level when all the effects were zero is indicated by  $b_0$ .

the level of the reference gene, *rm*, as described by Pfaffl (28). The relative expression (RE) was calculated from the amplification efficiencies of the *cntB* assay ( $E_{cntB}$ ) and the 16S rRNA gene assay ( $E_{rm}$ ) and the crossing point deviation ( $\Delta C_p$ ) of the unknown sample compared with a calibrator sample, as follows:  $RE = (1 + E_{cntB})^{\Delta C_{p_{cntB}}(\text{calibrator-unknown sample})} / (1 + E_{rm})^{\Delta C_{p_{rm}}(\text{calibrator-unknown sample})}$ .

The extracellular BoNT/B concentration was determined by an ELISA with polyclonal antibodies specific for the type B BoNT from rabbits (Metabio, Inc., Madison, Wis.), as described in the accompanying paper (19). Samples were collected at the same time as the samples used for RNA extraction in the late stationary growth phase. BoNT/B standards were included on each plate at the following concentrations: 1, 0.9, 0.8, 0.7, 0.6, 0.5, 0.4, and 0.3 ng/ml. Casein buffer was included as a negative control on each plate. Samples from both growth experiments for each medium combination were analyzed by ELISA, and an average value and standard deviation were calculated. For each sample the extracellular toxin concentration was correlated with the total cell mass by dividing the concentration by the maximum OD<sub>620</sub> value.

**Statistical analysis.** We constructed six regression models in order to obtain a quantitative description of how the explaining variables CO<sub>2</sub>, NaCl, and NaNO<sub>2</sub> are related to six outcome variables: LPD, growth rate, relative expression of *cntB* in the three different growth phases, and the extracellular BoNT/B concentration. For all six outcome variables, we used all linear, quadratic, and second-order interaction terms; i.e., we supposed that the outcome, except for independent and normally distributed statistical errors, was  $b_0 + b_1 \cdot [\text{CO}_2] + b_2 \cdot [\text{NaCl}] + b_3 \cdot [\text{NaNO}_2] + b_{11} \cdot [\text{CO}_2]^2 + b_{22} \cdot [\text{NaCl}]^2 + b_{33} \cdot [\text{NaNO}_2]^2 + b_{12} \cdot [\text{CO}_2] \cdot [\text{NaCl}] + b_{13} \cdot [\text{CO}_2] \cdot [\text{NaNO}_2] + b_{23} \cdot [\text{NaCl}] \cdot [\text{NaNO}_2]$ , and from this starting point the best-fit model with all *bs* significant was selected (7). We ended up with a smaller model (some *bs* were zero) when insignificant terms were removed. Note how the coefficients were constructed; the covariates were numbered 1, 2, and 3, and the coefficient before covariate 1 times covariate 2 was designated  $b_{12}$ , for example. To make estimates of the coefficients easier to compare, all values for CO<sub>2</sub>, NaCl, and NaNO<sub>2</sub> were divided by the largest values before they were put into the regression; i.e., the values for NaCl were divided by 2.5, the values for NaNO<sub>2</sub> were divided by 75, and the values for CO<sub>2</sub> were divided by 70. For the analysis of relative expression and the extracellular BoNT/B concentration, the logarithm of the value was used as the dependent variable, since this brought the data closer to the assumption of normal distribution and of equal variance of the error terms.

## RESULTS

**Effect on LPD and growth rate.** Various concentrations of carbon dioxide (10, 35, and 70% [vol/vol]), sodium chloride (0, 1.25, and 2.5% [wt/vol]), and sodium nitrite (0, 37.5, and 75 ppm) were added to TPY-C medium. *C. botulinum* Eklund 2B was able to grow in 21 of the 27 factorial-designed medium combinations (Table 1). The following model was used to quantitatively describe the interaction effects on LPD:  $LPD = b_0^i + b_{13}^i \cdot [\text{CO}_2] \cdot [\text{NaNO}_2] + b_{22}^i \cdot [\text{NaCl}]^2 + \text{error}$  (see Table 2 for the *b* values). The errors in the different measurements

were assumed to be independent Gaussian-distributed random variables with mean zero and constant variance. In the model, significant effects on the LPD were found for the quadratic term for NaCl and the interaction between NaNO<sub>2</sub> and CO<sub>2</sub> (Table 2). However, the effect of the interaction (50) (Table 2) was about twice that of the effect of NaCl (21) (Table 2). The LPD was extended with increasing concentrations of NaNO<sub>2</sub> and CO<sub>2</sub>. At 10 and 35% CO<sub>2</sub> the LPD was the same order of magnitude, whereas at 70% CO<sub>2</sub> and 75 ppm NaNO<sub>2</sub> the LPD was almost three times longer (69.0 h at 70% CO<sub>2</sub> compared with 24.5 h at 10% CO<sub>2</sub>).

**In the statistical analysis of the effect on the specific growth rate ( $\mu$ ) the following model was used:**  $\mu = b_0^{ii} + b_3^{ii} \cdot [\text{NaNO}_2] + b_{13}^{ii} \cdot [\text{CO}_2] \cdot [\text{NaNO}_2] + b_{22}^{ii} \cdot [\text{NaCl}]^2 + b_{11}^{ii} \cdot [\text{CO}_2]^2 + \text{error}$ . All of the preservatives investigated decreased the growth rate. However, the effects could not simply be added due to the positive interaction between NaNO<sub>2</sub> and CO<sub>2</sub>. The growth rate decreased (negative effect in Table 2) with increasing salt concentration, and the most significant effect was observed at 2.5% NaCl (Table 1). Furthermore, the maximum OD<sub>620</sub> value decreased as the NaCl concentration increased. For NaNO<sub>2</sub>, the same trend was observed; there was slower growth and a reduction in the maximum OD<sub>620</sub> value as the NaNO<sub>2</sub> concentration increased at both 10 and 70% CO<sub>2</sub>. These results agree with what was previously determined about the effects of NaCl and NaNO<sub>2</sub> on growth (16). However, at 35% CO<sub>2</sub> there was instead a small increase in both the growth rate and the OD<sub>620</sub>. The highest CO<sub>2</sub> concentration, 70%, was found to inhibit growth in all medium combinations compared with the growth in the other gas mixtures.

**Effect on *cntB* expression.** *cntB* expression was monitored three times during growth: in the middle of the exponential phase, in the late exponential phase, and in the late stationary phase (Table 3 and Fig. 1). The level of expression of the reference gene was found to be constant in all growth phases and media, as shown by stable crossing point values (data not shown). In the statistical analysis of the effect on *cntB* expression, the three growth phases were analyzed individually, resulting in the following three models: for the mid-exponential phase,  $\log(\text{RE}) = b_0^{iii} + b_{11}^{iii} \cdot [\text{CO}_2]^2 + b_{22}^{iii} \cdot [\text{NaCl}] + \text{error}$ ; for the late exponential phase,  $\log(\text{RE}) = b_0^{iv} + b_3^{iv} \cdot [\text{NaNO}_2] + b_{11}^{iv} \cdot [\text{CO}_2]^2 + b_{33}^{iv} \cdot [\text{NaNO}_2]^2 + \text{error}$ ; and for the late stationary phase,  $\log(\text{RE}) = b_0^v + b_1^v \cdot [\text{CO}_2] + b_2^v \cdot [\text{NaCl}] + \text{error}$ . In general, most combinations resulted in maximum *cntB* expression in the late exponential growth phase. Statistical analysis showed that NaCl had a significant effect on *cntB* transcription in the middle of the exponential growth phase and in the late stationary growth phase (Table 4). This was most obvious when the growth medium had been supplemented with 2.5% NaCl in the presence of both 10 and 35% CO<sub>2</sub>. In these cases the *cntB* mRNA level continued to increase after the late exponential phase and reached a maximum in the late stationary growth phase. In the presence of 70% CO<sub>2</sub> the maximum *cntB* mRNA level was again observed in the late exponential phase, but the level was much higher than the previous level (Fig. 1C).

Carbon dioxide had a significant effect on *cntB* expression in all three growth phases. Overall, the *cntB* mRNA level increased as the CO<sub>2</sub> concentration increased, and the highest levels were detected with the 70% CO<sub>2</sub> atmosphere. In the



TABLE 3. Interaction effects of NaCl, NaNO<sub>2</sub>, and CO<sub>2</sub> on the relative expression of the type B neurotoxin gene and the extracellular neurotoxin concentration

Expt	Exptl design			Relative expression of type B neurotoxin gene <sup>a</sup>			Extracellular neurotoxin concn (ng · ml <sup>-1</sup> · unit of OD <sub>620</sub> <sup>-1</sup> ) <sup>a</sup>
	CO <sub>2</sub> concn (%, vol/vol)	NaCl concn (%, wt/vol)	NaNO <sub>2</sub> concn (ppm)	Mid-exponential phase	Late exponential phase	Late stationary phase	
1	10	0	0	1.0, 1.0	2.9, 2.0	0.2, 0.7	25.7, 27.7
2	10	0	37.5	0.6, 0.4	0.4, 0.6	0.4, 0.3	28.9, 32.6
3	10	0	75	0.4, 0.4	0.9, 0.5	0.3, 0.4	21.7, 38.3
4	10	1.25	0	0.7, 0.9	1.0, 1.2	0.7, 1.0	20.9, 43.7
5	10	1.25	37.5	1.1, 0.8	0.8, 1.3	0.9, 1.0	40.4, 71.2
6	10	1.25	75	1.1, 0.9	0.8, 0.8	0.8, 0.6	35.2, 55.2
7	10	2.5	0	1.5, 1.3	1.3, 1.4	1.5, 1.8	37.9, 63.8
8	10	2.5	37.5	NG <sup>b</sup>	NG	NG	NG
9	10	2.5	75	NG	NG	NG	NG
10	35	0	0	0.8, 0.8	2.5, 1.2	0.4, 0.5	27.8, 47.9
11	35	0	37.5	0.6, 0.5	1.3, 0.9	1.0, 0.7	37.7, 53.9
12	35	0	75	0.6, 0.6	1.4, 0.8	0.8, 0.6	19.1, 30.9
13	35	1.25	0	1.2 <sup>c</sup>	2.7 <sup>c</sup>	0.8 <sup>c</sup>	27.9 <sup>c</sup>
14	35	1.25	37.5	1.0, 2.1	1.4, 1.4	2.3, 1.8	30.0, 44
15	35	1.25	75	NG	1.4, 1.6	1.1, 1.6	34.1, 54.8
16	35	2.5	0	2.2 <sup>c</sup>	2.7 <sup>c</sup>	3.3 <sup>c</sup>	64.2 <sup>c</sup>
17	35	2.5	37.5	NG	NG	NG	NG
18	35	2.5	75	NG	NG	NG	NG
19	70	0	0	6.6 <sup>c</sup>	12.3 <sup>c</sup>	2.0 <sup>c</sup>	126.2 <sup>c</sup>
20	70	0	37.5	2.4 <sup>c</sup>	9.0 <sup>c</sup>	1.0 <sup>c</sup>	104.2 <sup>c</sup>
21	70	0	75	3.9 <sup>c</sup>	5.4 <sup>c</sup>	1.6 <sup>c</sup>	87.5 <sup>c</sup>
22	70	1.25	0	4.2, 4.2	9.4, 12.0	3.0, 4.6	100.7, 180.0
23	70	1.25	37.5	1.3, 0.6	3.0, 4.7	3.7, 2.5	100.4, 152.4
24	70	1.25	75	2.6, 2.9	9.7, 8.1	4.5, 7.8	122.6, 182.0
25	70	2.5	0	6.3, 6.1	8.1, 10.1	4.0, 2.8	68.8, 120.8
26	70	2.5	37.5	NG	NG	NG	NG
27	70	2.5	75	NG	NG	NG	NG

<sup>a</sup> Data obtained from two experiments unless indicated otherwise.<sup>b</sup> NG, no growth.<sup>c</sup> Data obtained from a single experiment.

three cases in which the medium was not supplemented with any NaCl or NaNO<sub>2</sub> and only the effect of CO<sub>2</sub> was monitored, the largest difference in expression was observed in the late exponential growth phase, and there was a fivefold increase between 10 and 70% CO<sub>2</sub>. This was confirmed by measuring the extracellular BoNT/B concentration by the ELISA in the late stationary growth phase. Nitrite, when studied alone (experiments 1 to 3, 10 to 12, and 19 to 21 in Table 3) had an inhibitory effect on *cntB* expression, especially in the exponential phases. However, the impact was strong enough to be visible in the statistical analyses only during the late exponential phase (Table 4).

**Effect on BoNT/B production.** The following model was used for statistical evaluation of BoNT/B production:  $\text{BoNT/B} = b_0^{vi} + b_{11}^{vi} \cdot [\text{CO}_2]^2 + \text{error}$ . Table 5 shows the values. A higher concentration of the toxin was detected at an elevated CO<sub>2</sub> concentration, and the highest concentrations were detected with the 70% CO<sub>2</sub> atmosphere. In the presence of 10% CO<sub>2</sub> when the medium was not supplemented with NaCl or NaNO<sub>2</sub>, the BoNT/B concentration was 27 ng · ml<sup>-1</sup> · unit of OD<sub>620</sub><sup>-1</sup>. The corresponding value in the presence of 70% CO<sub>2</sub> was 126 ng · ml<sup>-1</sup> · unit of OD<sub>620</sub><sup>-1</sup>; thus, there was a fivefold increase in the level of extracellular toxin.

## DISCUSSION

In this investigation, we studied the effects of three food preservatives that are commonly used to control *C. botulinum*

in foods (carbon dioxide, sodium chloride, and sodium nitrite) on the *cntB* expression of the nonproteolytic strain *C. botulinum* Eklund 2B. Six statistical models were constructed to quantify and predict the interaction effects of these selected food preservatives on LPD, growth rate, relative *cntB* expression, and neurotoxin production.

The growth of *C. botulinum* and *cntB* expression were influenced by addition of CO<sub>2</sub>, NaCl, and NaNO<sub>2</sub> to TPY-C medium, both when the compounds were added individually and when they were added in combination. The stimulating effect of CO<sub>2</sub> on *cntB* expression could clearly be seen at all stages of growth. NaCl also appeared to have a stimulating effect, at least in the mid-exponential and late stationary phases. The impact of CO<sub>2</sub> was also seen in BoNT/B production (Table 5). The positive effect on toxin production was clear, unlike the effects of the other factors. The differences between the effects on toxin expression and toxin production can be explained by the role that factors other than the growth environment play in neurotoxin formation. For the most part the amount of neurotoxin formed can be predicted from the relative expression ratio in the late exponential phase. Including the other growth phases does not improve the analysis significantly (data not shown).

The combination consisting of 2.5% NaCl and 37.5 or 75 ppm of NaNO<sub>2</sub> totally inhibited growth in either of the CO<sub>2</sub> atmospheres investigated. Furthermore, we found that addition of NaNO<sub>2</sub> repressed the expression of *cntB*, which is in

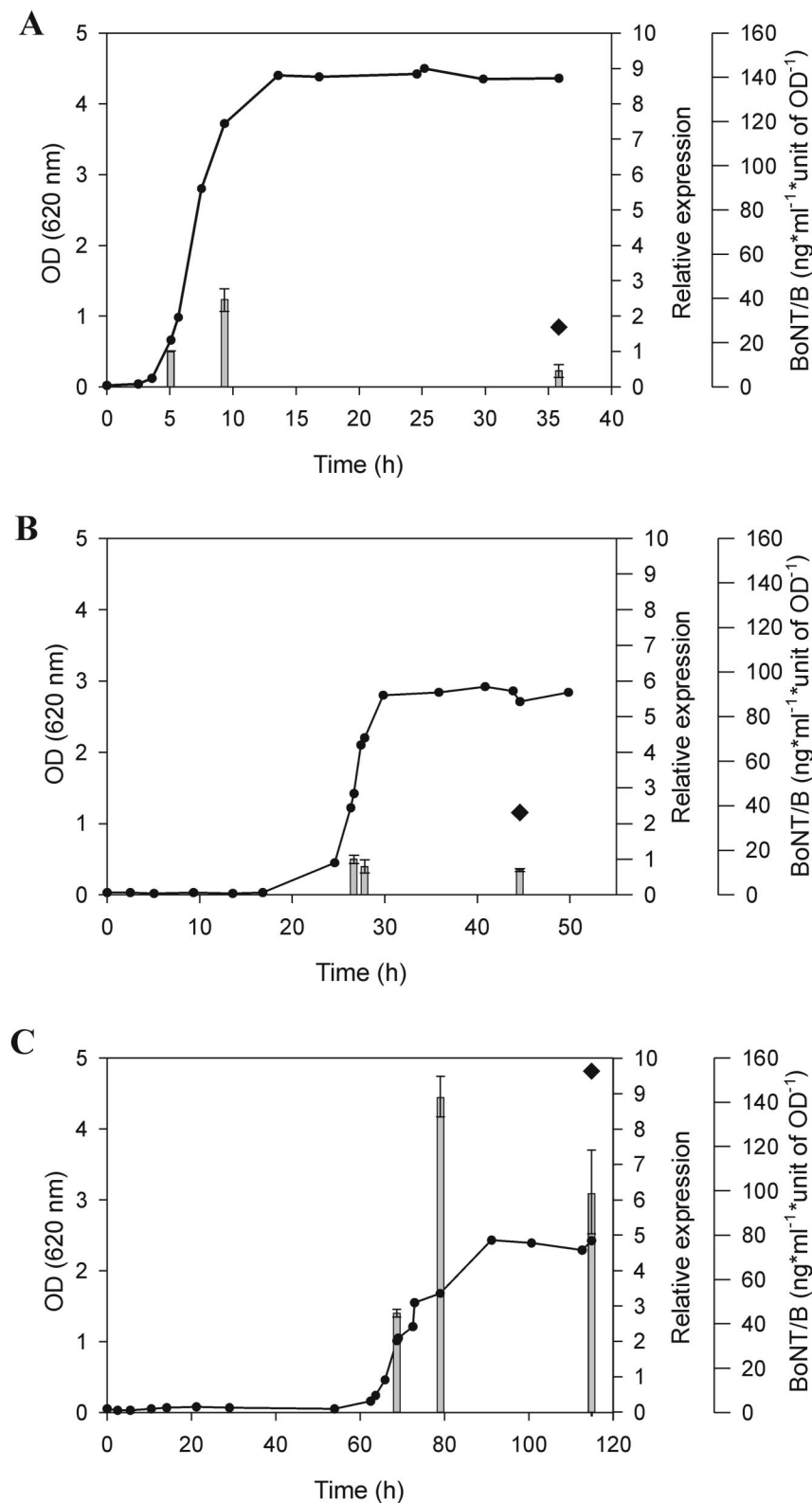


FIG. 1. Effects of carbon dioxide, sodium chloride, and sodium nitrite on LPD, growth, relative *cntB* expression, and extracellular BoNT/B formation in *C. botulinum* Eklund 2B. The concentrations of the different preservatives were as follows: 10% carbon dioxide, 0% sodium chloride, and 0 ppm of sodium nitrite (A); 10% carbon dioxide, 1.25% sodium chloride, and 75 ppm of sodium nitrite (B); and 70% carbon dioxide, 1.25% sodium chloride, and 75 ppm of sodium nitrite (C). ●, growth curve as determined by measurement of OD<sub>620</sub>; bars, relative expression of *cntB*; ◆, concentration of extracellular BoNT/B. The values are averages and standard deviations based on two independent growth experiments.

TABLE 4. Effects on relative neurotoxin expression during growth of *C. botulinum* Eklund 2B<sup>a</sup>

Phase	Coefficient	Effect	95% confidence interval
Mid-exponential	$b_0^{iii}$	-0.6	-0.8, -0.4
	$b_2^{iii}$	0.9	0.5, 1.2
	$b_{11}^{iii}$	1.6	1.3, 1.9
Late exponential	$b_0^{iv}$	0.3	0.1, 0.5
	$b_3^{iv}$	-2.1	-3.2, -1.2
	$b_{11}^{iv}$	2.1	1.8, 2.4
	$b_{33}^{iv}$	1.6	0.6, 2.6
Late stationary	$b_0^v$	-1.2	-1.4, -0.9
	$b_1^v$	1.5	1.2, 1.9
	$b_2^v$	1.4	1.1, 1.7

<sup>a</sup> The effect of CO<sub>2</sub> is indicated by 1, the effect of NaCl is indicated by 2, and the effect of NaNO<sub>2</sub> is indicated by 3, so that  $b_1$  is the effect of [CO<sub>2</sub>],  $b_{11}$  is the effect of [CO<sub>2</sub>],  $b_2$  is the effect of [NaCl],  $b_3$  is the effect of [NaNO<sub>2</sub>], and  $b_{33}$  is the effect of [NaNO<sub>2</sub>]<sup>2</sup>. The level when all the effects were zero is indicated by  $b_0$ .

agreement with findings described in the accompanying paper (19). However, BoNT/B production was unchanged, which may be explained by cell death in the culture and release of BoNT/B into the culture fluid. Addition of NaCl stimulated *cntB* expression, both in the middle of the exponential growth phase and in the late stationary growth phase. As a result of the higher gene expression, the concentration of BoNT/B was higher (51 ng · ml<sup>-1</sup> · unit of OD<sub>620</sub><sup>-1</sup>) than the concentration in the medium with no NaCl (27 ng · ml<sup>-1</sup> · unit of OD<sub>620</sub><sup>-1</sup>). At the same time, we found that NaCl extended the LPD and reduced the specific growth rate. This may indicate that *cntB* expression is induced when the bacteria are in a saline environment. NaCl is one of the most commonly used and important food preservatives for control of *C. botulinum* as it lowers the water activity in foods (15). In general, nonproteolytic strains of *C. botulinum* can be totally inhibited by 5% (wt/vol) salt under otherwise optimal growth conditions (15). However, foods containing high concentrations of salt are not especially popular among consumers. Therefore, a combination of NaCl and NaNO<sub>2</sub> is often used to reduce the amount of NaCl needed to inhibit *C. botulinum*.

When 1.25% NaCl and 75 ppm NaNO<sub>2</sub> were combined in the growth medium (Fig. 1), we found that the gene expression was still pronounced in all three growth phases and that the extracellular BoNT/B concentration was still unchanged (45 ng · ml<sup>-1</sup> · unit of OD<sub>620</sub><sup>-1</sup>). This combination of NaCl and NaNO<sub>2</sub> was also found to increase the LPD sixfold compared with the LPD obtained with the medium to which no preservatives were added. The increase in the LPD was probably an effect of the NaNO<sub>2</sub> added to the medium, as the LPD was the same order of magnitude as the LPD observed when NaNO<sub>2</sub> alone was used. The growth rate seemed to be influenced more by the increasing NaCl concentration. Thus, the concentration of the extracellular BoNT/B remained unchanged, regardless of the inhibition of the growth of *C. botulinum*.

Generally, the interaction of NaNO<sub>2</sub> and CO<sub>2</sub> significantly increased the LPD and reduced both the growth rate and the maximum OD<sub>620</sub>. However, in the presence of 35% CO<sub>2</sub>, growth was observed earlier and was somewhat faster than the growth in medium with 10 or 70% CO<sub>2</sub>. This was also observed when we used some of the other media containing NaNO<sub>2</sub>; e.g., 35% CO<sub>2</sub> stimulated growth by increasing both the growth

rate and the maximum OD<sub>620</sub> compared with the growth rates and the maximum OD<sub>620</sub> values obtained with the other CO<sub>2</sub> concentrations. Similar findings (i.e., that CO<sub>2</sub> can either stimulate or inhibit growth depending on the concentration in the gas atmosphere) have been reported previously for microbiological growth medium (8). Fernandez et al. (8) observed a substantial inhibitory effect on the growth of *C. botulinum* with 90% CO<sub>2</sub>, moderate inhibition of growth with 50% CO<sub>2</sub>, and stimulation of growth with 5% CO<sub>2</sub>. In addition to the stimulatory and inhibitory effects on vegetative growth, CO<sub>2</sub> is also known to act as a spore germinant in *C. botulinum* (9). It is important to take these issues into consideration as replacing the air in the headspace in modified-atmosphere packaging with CO<sub>2</sub> can result in a hazard rather than protection with regard to food safety.

Furthermore, CO<sub>2</sub> in the gas atmosphere was found to have the most dominant effect on *cntB* expression when all three growth phases were considered. Overall, expression of *cntB* was stimulated in the presence of elevated CO<sub>2</sub> concentrations; the highest levels were detected in a 70% CO<sub>2</sub> atmosphere. In some combinations a fivefold increase in relative expression was observed when we compared 10% CO<sub>2</sub> with 70% CO<sub>2</sub>. This finding was confirmed when we analyzed the extracellular BoNT/B concentration in the late-stationary-phase cell culture fluid, in which the highest concentrations of the toxin were observed with the 70% CO<sub>2</sub> atmosphere. The concentrations were found to be four- to fivefold higher in this atmosphere than in the presence of 10% CO<sub>2</sub>. An elevated CO<sub>2</sub> concentration has been reported to induce the expression of genes involved in toxin production in *Bacillus anthracis* (2) and *Vibrio cholerae* (31). It was recently found that in the more closely related organism *C. difficile* an elevated bicarbonate concentration in peptone-yeast extract broth increased the toxin yield 10-fold (14). In our growth experiments the pH of the growth media was reduced from pH 7 to pH 5 (data not shown). As CO<sub>2</sub> in the gas phase is more easily dissolved at a lower pH and dissociates into bicarbonate in a liquid medium, the stimulating effect that we observed on BoNT/B formation in this study could be explained by an elevated bicarbonate concentration in the TPY-C medium. In the bacterial cell an elevated concentration of bicarbonate influences several biosynthetic reactions, including biotin-dependent carboxylation. Karlsson et al. (14) suggested a possible link between carboxylation and amino acid synthesis through  $\alpha$ -ketobutyrate, a precursor in isoleucine biosynthesis. In some anaerobic bacteria reductive carboxylation occurs during the synthesis of  $\alpha$ -ketobutyrate from propionyl coenzyme A. In addition, it has been reported that production of BoNT is regulated by different amino acids (6, 16, 25). This may provide a possible explanation.

TABLE 5. Effects on extracellular BoNT/B concentration of *C. botulinum* Eklund 2B<sup>a</sup>

Coefficient	Effect	95% confidence interval
$b_0^{vi}$	29	18, 40
$b_{11}^{vi}$	90	70, 110

<sup>a</sup> The effect of CO<sub>2</sub> is indicated by 1, the effect of NaCl is indicated by 2, and the effect of NaNO<sub>2</sub> is indicated by 3, so that  $b_{11}$  is the effect of [CO<sub>2</sub>]<sup>2</sup>. The level when all the effects were zero is indicated by  $b_0$ .

tion for both the increased level of *cntB* expression and the increased extracellular BoNT/B concentration that we observed in this study.

In conclusion, CO<sub>2</sub> is often used to control the growth of microorganisms in ready-to-eat foods. However, our work showed that even though a high concentration of CO<sub>2</sub> decreased the growth rate, the expression and production of toxin were greatly increased. This means that the risk of botulism may actually be increased, instead of reduced, by the use of modified-atmosphere packaging. Further studies of the interaction effects of food preservatives on neurotoxin expression and production in different foods by *C. botulinum* may provide new and important information concerning food safety and may help us formulate new alternative methods for food preservation.

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