Expression of botulinum neurotoxins A and E, and associated non-toxin genes, during the transition phase and stability at high temperature: analysis by quantitative reverse transcription-PCR

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Production of botulinum neurotoxin A (BoNT/A) and associated non-toxic proteins (ANTPs), which include a non-toxic non-haemagglutinin (NTNH/A) as well as haemagglutinins (HAs), was found previously to be dependent upon an RNA polymerase alternative sigma factor (BotR/A). Expression of the *botR/A*, *bont/A* and *antp* genes, monitored by reverse transcription and real-time PCR analysis, occurred concomitantly at the transition between the exponential and stationary growth phases of *Clostridium botulinum* A. The *botR/A* expression level was about 100-fold less than those of the *bont/A* and *antp* genes. Therefore, BotR/A is an alternative sigma factor controlling the botulinum A locus genes during the transition phase. The highest toxin concentration was released into the culture supernatant 12 h after maximum expression of the *botR/A*, *bont/A* and *antp* genes, without any apparent bacterial lysis. Toxin levels were then stable over 5 days in cultures at 37 °C, whereas a dramatic decrease in lethal activity was observed between 24 and 48 h in cultures at 44 °C. High temperature did inhibit transcription, since expression levels of the *botR/A*, *bont/A* and *antp* genes were similar in cultures at 37 and 44 °C. However, incubation at 44 °C triggered a calcium-dependent protease that degraded BoNT/A and NTNH/A, but not HAs. In *C. botulinum* E, which contains no gene related to *botR*, the *bont/E* and *p47* genes were also expressed during the transition phase, and no protease activation at 44 °C was evident.

INTRODUCTION

Botulinum neurotoxins (BoNTs) are the most potent toxins known, and seven BoNT toxinotypes (A to G) have been recognized based upon their antigenic properties. They are produced by distinct strains of *Clostridium botulinum* and by atypical strains of other *Clostridium* species that display heterogeneous bacteriological characteristics. BoNTs are synthesized as single-chain proteins (approx. 150 kDa) and exported from the bacteria by an unknown mechanism. Each toxinotype is proteolytically cleaved into a heavy (H) chain (approx. 100 kDa) and a light (L) chain (approx. 50 kDa), which remain linked by a disulfide bridge. The H chain recognizes through its C-terminal part a specific cell-surface receptor on nerve endings, and then facilitates toxin uptake into cells by receptor-mediated endocytosis. The L chain translocates into the cytosol of motoneurons, where it gains access to substrate. L chains are zinc-dependent proteases that specifically cleave one of the three soluble N-ethylmaleimide-sensitive factor (NSF) attachment protein receptors (SNARE), thus blocking evoked acetylcholine

release at the skeletal neuromuscular junction (reviewed by Humeau *et al.*, 2000; Meunier *et al.*, 2002; Schiavo *et al.*, 2000).

BoNTs and associated non-toxic proteins (ANTPs) form complexes of various sizes (300–900 kDa). ANTPs consist of a non-toxic non-haemagglutinin (NTNH) component, the size of which is similar to BoNT, and haemagglutinin (HA) components. ANTPs spontaneously associate with BoNT at low pH by non-covalent bonds and form stable complexes that dissociate at high pH (pH 8 and above) (reviewed by Popoff & Marvaud, 1999). The precise role of ANTPs and botulinum complexes remains unclear, but they possibly protect BoNT against acidic pH and proteolytic degradation during passage through the stomach and intestine. ANTPs might also be involved in transporting BoNT across the intestinal barrier (Fujinaga *et al.*, 1997, 2000).

In *C. botulinum* A, *bont* and *antp* genes are clustered in a chromosomal DNA segment called the botulinum locus (reviewed by Henderson *et al.*, 1997; Popoff & Marvaud, 1999). These genes are organized in two operons. The first operon (*ntnh/A-bont/A*), which is located at the 3' end of the botulinum locus, encompasses the *bont* gene immediately preceded by the *ntnh/A* gene. Both genes are transcribed in

Abbreviations: ANTP, associated non-toxic protein; BoNT, botulinum neurotoxin; HA, haemagglutinin; Hc, heavy chain; Lc, light chain; MLD, mouse lethal dose; NTNH, non-toxic non-haemagglutinin.

the same orientation, and the organization of this operon is highly conserved in all *C. botulinum* types. The second operon contains the *ha* genes and differs slightly between various toxinotypes. In *C. botulinum* A, the *has* operon contains successive genes for the 34 kDa (*ha34*), 17 kDa (*ha17*), and 70 kDa (*ha70*) haemagglutinins (Henderson *et al.*, 1996). Genes of the *ha* operon are transcribed in an opposite orientation versus the *ntnh/A-bont/A* genes. The *botR* gene, which has features of a DNA-binding protein (i.e. highly basic isoelectric point and helix–turn–helix motif), is localized at the 5' end of the botulinum locus in *C. botulinum* types C and D and between the two operons in *C. botulinum* types A, B and F (Henderson *et al.*, 1997; Popoff & Marvaud, 1999). *botR/A* from *C. botulinum* type A has been characterized as a transcriptional activator of *bont* and *ha* genes based on *botR/A* overexpression or partial inhibition by antisense mRNA in *C. botulinum* (Marvaud *et al.*, 1998b). Recently, BotR/A was found to be an alternative sigma factor of RNA polymerase, which forms, with *Clostridium tetani* TetR, *Clostridium difficile* TxrR and *Clostridium perfringens* UviA, a new subgroup of the sigma 70 family of RNA polymerase involved in controlling clostridial toxin genes (Raffestin *et al.*, 2005).

Genetic organization of the botulinum locus is slightly different in *C. botulinum* E strains, which are non-proteolytic bacteria unable to activate BoNT/E. The BoNT/E locus contains no gene related to *botR*, but immediately upstream of *ntnh/E-bont/E* and in a position equivalent to that of *botR/A* lies a gene encoding a 47 kDa protein (*p47*). The second operon encompasses *orfX1* and *orfX2* instead of *ha* genes (Hatheway, 1993; Popoff & Marvaud, 1999).

In this study, we report that the expression of *botR/A* is concomitant with that of *bont/A* and *antp*. This expression occurs during the transition from the exponential to the stationary growth phase, as assessed by quantitative reverse transcription-PCR (RT-PCR). In *C. botulinum* E, *bont/E* and *p47* are also expressed during the transition phase. In addition, high temperature (44 °C) does not act as a BotR/A-mediated regulation signal, but activates a protease which degrades BoNT/A and NTN/A.

METHODS

Bacterial strains and cultures. *C. botulinum* type A strains Hall A and NCTC 2916, and *C. botulinum* type E strain HV from the National Reference Centre for anaerobic bacteria (Institut Pasteur), were grown in TGY broth (pH 7.5) containing trypticase (Trypticase-Peptone BBL, BD Biosciences; 30 g l⁻¹), yeast extract (Bacto Yeast Extract, BD Biosciences; 20 g l⁻¹), glucose (5 g l⁻¹) and cysteine.HCl (0.5 g l⁻¹) under anaerobic conditions (N₂/CO₂/H₂; 90:5:5, by vol. at 37 °C. Spores were germinated by heating (10 min, 95 °C) and subsequent inoculation into TGY.

Kinetic experiments were performed by incubating 500 ml TGY inoculated with either 10 ml of an overnight preculture (non-sporulated cultures) or 10 ml of a culture containing 0.5 ml heat-activated (10 min, 95 °C) spore suspension (about 2 × 10⁷ spores ml⁻¹). Bacterial growth was monitored as OD₆₀₀. Culture samples

(5–10 ml) were removed at 3, 6, 9, 10, 11, 12, 24 and 48 h. Bacteria were collected by centrifugation and stored at –20 °C until RNA extraction, and the supernatants were stored at 4 °C for mouse bioassays. Total DNA was prepared as previously described (Marvaud *et al.*, 1998b), and at least three experiments were independently performed for each strain and condition.

Total RNA extraction. Bacterial pellets were washed and suspended in 1 ml distilled water treated with diethyl pyrocarbonate (DEPC, Sigma). For total RNA extraction, 400 µl bacterial suspension was used when the culture OD₆₀₀ was below 0.4, 200 µl when the OD₆₀₀ ranged between 0.4 and 2, and 100 µl otherwise. Bacteria were centrifuged at 4 °C (7500 g, 10 min) and total RNA was extracted using a RNeasy Mini kit (Qiagen) according to the manufacturer's guidelines. Contaminating genomic DNA was degraded by treating the 60 µl RNA sample with 2 U DNA-free DNase (Ambion) and 1 × reaction buffer. The reaction mixture was incubated at 37 °C for 30 min and the DNase was removed by adding DNase Inactivation Reagent (DNase-free, 0.1 ×) (Ambion) to the mixture. Total RNA concentrations were determined by measuring A₂₆₀ and the formula 1 A₂₆₀ unit = 40 µg RNA. RNA purity was monitored using the ratio A₂₆₀/A₂₈₀ (between 1.8 and 2.2) and real-time PCR was then done on the RNA samples, which were immediately used as a template in cDNA synthesis.

Reverse transcription. First-strand cDNA synthesis was performed in triplicate with random hexamers PDN6 and the M-MLV kit (Invitrogen) in a 20 µl final volume according to the handbook protocol. The reaction mixture contained 1 µg total RNA, 25 ng PDN6 µl⁻¹ (Roche Boehringer), 10 mM each nucleotide dATP, dTTP, dCTP and dGTP (Amersham Biosciences), 40 U RNA guard RNase inhibitor (Amersham Biosciences), 0.01 M DTT (Invitrogen), 1 × first-strand buffer (Invitrogen), and 200 U M-MLV reverse transcriptase (Invitrogen). Before RT enzyme was added, the reaction mixture was heated to 65 °C for 5 min and thereafter chilled on ice. It was then incubated for 10 min at 25 °C and 50 min at 37 °C. The reaction was inactivated by heating at 70 °C for 15 min. The three replicates were pooled and aliquoted. An aliquot was diluted 10-fold in distilled DEPC-treated water. cDNA solutions were stored at –20 °C before PCR amplification.

Quantitative real-time PCR assay. The real-time PCR assay was based on the double-stranded DNA-specific dye SYBR Green I methodology. The primers used are listed in Table 1. A primer pair specific for each of six genes in the botulinum gene locus (*bont/A*, *ntnh/A*, *ha17*, *ha34*, *ha70* and *botR/A*) or the 16S rRNA gene was designed from the Hall A genome sequence (<http://www.ncbi.nlm.nih.gov>) with Beacon Designer version 2.0 (Bio-Rad). Primers were also designed for *bont/E*, *p47* and the 16 rRNA gene from *C. botulinum* E (X62089 for *bont/E*, D88418 for *p47* and L37592 for 16 rRNA).

Reverse-transcribed cDNA samples (1 µl) were subjected to PCR amplification in 24 µl ready-to-use iQ SYBR Green Supermix (Bio-Rad, 2 ×; 1.25 U iQ Taq DNA polymerase, 0.4 mM each dNTP, 6 mM MgCl₂, 20 nM fluorescein, SYBR Green I) containing 0.5 µM each primer. The reactions were cycled in an iQ iCycler apparatus (Bio-Rad) using the following parameters: Taq polymerase activation at 95 °C for 10 min then 40 cycles at 95 °C for 15 s, 55 °C for 15 s and 72 °C for 15 s with fluorescence measurement at the end of extension, and finally a cooling step at 55 °C for 1 min before the melting curve programme (55 °C to 95 °C with a heating rate of 0.5 °C per 10 s and continuous fluorescence measurement).

Quantitative analysis was performed using the iCycler iQ Real-Time PCR Detection System Software Version 3.0 (Bio-Rad). Amplification plots displaying fluorescence for each well at every cycle were obtained. Each sample was amplified in triplicate. The background fluorescence

Table 1. Primers used to amplify the internal fragments of the target genes

Gene	Mol% G + C	Primer*	Sequence (5'–3')	Product length (bp)	T _m (°C)	PCR efficiency (%)
<i>bont/A</i>	26	F	AATGGTTATGGCTCTACTCAATAC	134	81	95
		R	GCTAATGTTACTGCTGGATCTG			
16 rRNA A gene	52	F	GCGTAGCCGACCTGAGAG	149	88.5	82
		R	ACAATCCGAAGACCTTCATCAC			
<i>botR/A</i>	21	F	AAAGTTGATTTGAGCAATTTCAATACAC	233	76.5	81
		R	ATTTTCTTTTGATCGTCAGGTAATATGG			
<i>ha17</i>	25	F	TCCAATAGATGCTACTGGTTTCC	150	77.5	89
		R	ATTCGGTAATAGGAGTAGTGGTTG			
<i>ha34</i>	28	F	AAGTCGTACAACAAGTGGATATG	148	78.5	84
		R	GTTAGAACTCCGTTTGAAGTATTG			
<i>ha70</i>	27	F	CTAATGCTCCTTCACCTGAATCTG	100	80	90
		R	ATGACACCTACTACTACAATTTCTTC			
<i>ntnh/A</i>	25	F	AATGTTGTAGTAGTTAGAGCTAG	107	81.5	89
		R	CTCAAAGATTCCGCATAATATC			
<i>bont/E</i>	25	F	AAGCAAAGTATGGATTAGATAAAGATG	114	77	89
		R	TAGTTCTGTAATCAAATTCGTAAG			
16 rRNA E gene	52	F	GGAGCGAACAGGATTAGATACC	110	85	88
		R	GGCGGAATACTTAATGCGTTAG			
<i>P47</i>	23	F	CAACTGATAATAATGATGGATACTTGTG	112	76	82
		R	ACTTCACTATTCTCTGATAAGATACTAC			

F, forward; R, reverse.

was automatically removed by setting a noise band. The threshold cycle (c_T) for each transcript was determined as the PCR cycle at which the fluorescence rose above background. Because the c_T is inversely proportional to the logarithm of the initial number of template molecules, the threshold cycle value was used in computation for quantification of gene expression. A standard curve consisting of the c_T versus log chromosomal DNA dilutions (corresponding to the log copy numbers) was generated by amplifying serial dilutions of chromosomal DNA corresponding to an unknown amount of amplicon. For each primer set, PCR was performed in parallel reactions using different amounts of Hall A chromosomal DNA (150 ng to 1.5 pg) and each cDNA sample in the same 96-well analysis. For each target gene, the software determined the unknown concentration in the cDNA samples by interpolating the c_T of the sample against the standard curve of known concentrations.

Expression levels of cDNA from the six botulinum locus genes were standardized against 16S rRNA gene expression. The 16S rRNA gene primer set was run concurrently with each set of experimental primers. At different points during the growth kinetics, relative expression of the six genes ($X_1, X_2, \dots, X_i, \dots, X_6$) compared to 16S rRNA gene expression (Y) was determined by the formula $X_i = X'_i / Y$. X'_i is the arbitrary genomic DNA dilution given by c_T values on a standard curve and corresponds to the amount of gene i transcripts in DNA samples.

The identity and specificity of PCR products were verified by high-resolution agarose gel electrophoresis, and a single PCR product migrating at the expected size was observed for each primer pair. In addition, an iQ iCycler melting curve confirmed the presence of a unique peak for the negative first derivative of the temperature versus fluorescence plotted against temperature (melt curve) with single product-specific melting temperatures (T_m). For this, fluorescence was continuously monitored in samples heated from 55 to 95 °C at a transition rate of 0.1 °C. In order to check for amplification of any

contaminating genomic DNA in the RNA sample after extraction or in the PCR mixture, DNase-treated RNA and sterile water were added to the PCR.

Mouse bioassay. Serial twofold dilutions of supernatant samples (0.5 ml) in 50 mM sodium phosphate buffer (pH 6.3) containing 0.2% (w/v) gelatin were injected intraperitoneally into mice weighing 18–20 g. For type E supernatant, the toxin was activated with trypsin (200 µg ml⁻¹, Sigma) for 15 min at room temperature prior to dilution. Three mice were used for each dilution. The mice were observed over 4 days, and the number that died was recorded, with results expressed as mouse lethal dose (MLD) per millilitre.

Toxin stability assay. One-millilitre aliquots of supernatant from a 24 h Hall culture were incubated at 44 °C for different time periods: 30 min, 3 h, 6 h and 24 h. The supernatant was also incubated with protease inhibitors: EDTA (0.5 and 1 mM), EGTA (5 and 1 mM), *o*-phenanthroline (5 mM), PMSF (1 mM), pepstatin (10 µg ml⁻¹) and benzamidine (1 mM) for 6 h at 44 °C. Toxin was then precipitated at pH 3.0 with 10 M H₂SO₄ and centrifuged 10 min at 7500 g and 4 °C. The precipitates were washed with distilled water and resuspended in 0.2 M phosphate buffer, pH 6.0. Protein concentration was determined by the Bradford method (Bio-Rad) using BSA as standard.

PAGE and Western blotting. Each precipitate (5 µg total protein) was separated by electrophoresis on SDS-polyacrylamide gels (10%). Proteins were transferred to PVDF membranes (Hybond-C, Amersham), which were blocked with 5% skimmed milk (w/v) in PBS (pH 7.2) and incubated 1 h at room temperature with rabbit antibodies (1/1000) against purified recombinant BoNT/A (Hc or Lc), NTN/A, HA34 or HA52 (processed form of HA70) (Tavallaie *et al.*, 2004). DNAs encoding BoNT/A Hc (amino acids 872–1296), Lc (amino acids 1–438), NTN/A (amino acids 783–1206), HA34 (amino acids 1–293) and HA52 (amino acids 203–626) were cloned

in pET28 vector in fusion with a N-terminal His₆-tag, and the recombinant proteins were purified on a cobalt column (Clontech) as previously described (Tavallaie *et al.*, 2004). After three washes in PBS/0.1% Tween 20, the blots were treated with horseradish peroxidase-conjugated protein A (Bio-Rad) and analysed by the enhanced chemiluminescence system (ECL, Amersham Pharmacia Biotech).

RESULTS

Bacterial growth and toxin production in *C. botulinum* A

Type A *C. botulinum* strains Hall and NCTC 2916 showed a typical growth curve as monitored by OD₆₀₀, which included a lag period, an exponential growth phase and a subsequent stationary phase characterized by a progressive decrease in OD₆₀₀. Termination of the exponential growth phase and entry into stationary phase (transition phase), which is characterized by the arrest of cell division and a progressive bacterial lysis, occurred about 10 h after initiation of the exponential phase (Fig. 1a, b). The lag phase was slightly longer at 5–6 h versus 2–3 h in non-sporulated cultures, but the growth curve was similar, although strain Hall showed a slower decrease in OD during the stationary phase in sporulated cultures compared to strain NCTC 2916 (Fig. 1b). Spores were determined by phase-contrast microscopy and expressed as a percentage of the total bacterial population. *C. botulinum* strain NCTC 2916 was selected since it yielded a high percentage of sporulation in our culture conditions. Spores of strain NCTC 2916 were detected within 10 h after inoculation, corresponding to the transition phase, and increased to 65% at 24 h and almost 100% at 48 h. In contrast, spores of strain Hall were not apparent until 32 h after inoculation of the culture and then increased to 28% at 48 h and reached only 85% at 156 h.

The production and secretion of BoNT/A in the culture supernatant, as tested by the mouse bioassay, was similar for both strains in sporulated and non-sporulated cultures. Low levels of toxicity (10 MLD ml⁻¹) were detected during entry into exponential growth. Lethal activity in the culture supernatant increased over time and reached 10³ MLD ml⁻¹ during mid-exponential growth, and 10⁴ MLD ml⁻¹ at the transition phase. The highest toxic value (10⁵ MLD ml⁻¹) was observed at 24 h and was stable for a period of at least 5 days (Fig. 1c, d, e, f). These results were in agreement with previous reports (Bradshaw *et al.*, 2004; Siegel & Metzger, 1979).

Relative expression of the type A botulinum locus genes by real-time PCR

Gene expression was assayed by mRNA reverse transcription and cDNA amplification with real-time PCR. The amplified products were quantified by measuring SYBR Green fluorescence incorporated into double-stranded DNA resulting from a single specific gene product as determined by melting temperature curves. For this purpose, a primer

pair was designed for each target gene in order to define a DNA fragment having a high *T_m*. Since the *C. botulinum* genome has a low G + C content, the *T_m* of the PCR products of the botulinum toxin locus genes ranged from 76.5 to 81 °C (Table 1). However, the *T_m* of the PCR product of the 16S rRNA gene, which has a greater G + C content (52 mol% versus 21–28 mol% for the botulinum toxin locus genes), was higher (88 °C). Despite the different *T_m* values, calibration assays of PCR amplification of each gene with *C. botulinum* Hall or NCTC 2916 genomic DNA showed a correlation coefficient above 0.995 over 40 amplification cycles, indicating a linear measurement of 15 pg to 150 ng DNA (data not shown). All culture samples submitted to RT and cDNA real-time PCR yielded DNA amplification within this linear range. In addition, RNA samples prior to reverse transcription treatment did not yield any significant PCR amplification, indicating that DNA contamination was negligible.

Quantitative expression of the 16 rRNA gene as calculated by fluorescence intensity per microgram of RNA was constant over bacterial growth, at least until 48 h, in sporulated and non-sporulated cultures. Therefore, the 16S rRNA gene was used as a calibrator gene. An equal amount of total mRNA from each sample was used for relative quantification of gene expression. The levels of each gene transcript were then normalized to the 16S rRNA gene. Transcript of each botulinum toxin locus gene was detectable in all stages of growth in both strains and in sporulated and non-sporulated cultures. However, the relative expression of *botR/A* in *C. botulinum* strains Hall and NCTC 2916 was approximately 100-fold less than that of the other botulinum toxin locus genes.

Relative quantification of gene expression within the botulinum toxin locus, versus that of the 16 rRNA gene, revealed a concomitant expression of the *botR/A*, *bont/A* and *antp* genes in sporulated and non-sporulated cultures (Fig. 2). The expression pattern over time was similar for all these genes in both strains. A low expression level was detected at the beginning of exponential growth, followed by a rapid increase in expression during late exponential growth. A maximum expression value was reached at the transition phase (10 h incubation) and then expression decreased during the stationary phase. Relative expression of each gene within the botulinum toxin locus was increased 100-fold in the transition phase versus levels in the early exponential phase. After 24 h incubation, relative gene expression decreased to levels found in the early growth phase. Thereafter, the expression remained at basal levels during stationary phase, with a slight transient increase at 48 h.

Expression kinetics for the botulinum toxin locus genes were the same in both strains. The most relevant feature was synchronous expression of the *botR/A*, *bont/A* and *antp* genes, which occurred primarily during the transition phase. However, the *bont/A* and *antp* genes showed different levels of relative expression. Fig. 3 shows quantification of the

Fig. 1. (a, b) Growth and sporulation kinetics of *C. botulinum* strain Hall (filled symbols) and NCTC 2916 (open symbols) in non-sporulated (a) and sporulated (b) cultures. Growth curves (circles) were determined by measurement of OD₆₀₀. The lag phase of cultures in sporulated cultures is not reported. Refractile spores were counted with a phase-contrast microscope and were expressed as percentage of the vegetative cell population (squares). Data are mean values from two independent experiments for each strain. (c–f) Production of botulinum neurotoxin (BoNT/A) in the extracellular medium during the growth of *C. botulinum* A strains Hall (c, d) and NCTC 2916 (e, f) in non-sporulated (c, e) and sporulated (d, f) cultures. Growth curve (◆) was determined by measuring OD₆₀₀. BoNT/A production was assayed with the mouse test (MLD ml⁻¹, ■). It was confirmed that lethal activity corresponded to BoNT/A by using specific neutralizing antibodies against BoNT/A. The lag phase of cultures in sporulated cultures is not reported. Note that BoNT/A was detected at a low level during entry into exponential growth, increased to a maximal value at 24 h and then remained constant, independent of sporulation. Data are mean values from two independent experiments for each strain and condition.

botulinum toxin locus gene expression versus the 16 rRNA gene obtained from three independent sporulated and non-sporulated cultures. In both strains and culture conditions, the relative expression of each botulinum toxin locus gene predominated at the transition phase. In the non-sporulated Hall strain, the relative levels of *bont/A* and *antp* transcripts at the transition phase were similar, whereas in sporulated cultures each *ha* gene was expressed in higher amounts (about twofold) compared to the *bont/A* and *ntnh/A* genes. In contrast, *C. botulinum* NCTC 2916 showed similar values of relative expression in both sporulated and

non-sporulated cultures. However, the botulinum toxin locus genes were expressed in different amounts: the relative expression of *bont/A* was approximately twice that of the *ntnh/A* gene, and *ha34* transcript levels were about twofold higher than those of *ha17* and *ha70* (Fig. 3).

BoNT/A synthesis is independent of high temperature

Since temperature is a common environmental factor controlling many bacterial toxin genes, we tested the effect of

Fig. 2. Relative expression of the botulinum locus genes [$\diamond$, *botR/A* ($\times 100$); $\square$, *bont/A*; $\triangle$, *NTNH/A*; $\bullet$, *ha34*; $\blacksquare$, *ha17*; $\blacklozenge$, *ha70*] versus the 16 rRNA gene during growth of *C. botulinum* A strains Hall (a, b) and NCTC 2916 (c, d) in non-sporulated (a, c) and sporulated (b, d) cultures. Growth curves ($\circ$) were determined by measurement of OD_{600} . Lag phase of the sporulated cultures is not reported. Data are mean values from three independent experiments for each strain and condition.

Fig. 3. Transcript levels from the botulinum locus genes versus the 16 rRNA gene at (from left to right, alternately black and white) early, mid-exponential, transition, and stationary growth phases at 24 h and 48 h for cultures of *C. botulinum* strains Hall (a, b) and NCTC 2916 (c, d), non-sporulated (a, c) and sporulated (b, d). The relative expression of *botR/A* is amplified 100-fold. The six botulinum locus genes were predominantly expressed during the transition phase. Data are means $\pm$ SE from three independent experiments for each strain and condition.

high temperature on expression of the botulinum toxin locus genes and BoNT/A production. Incubation of the Hall strain in TGY broth at 44 °C under anaerobic conditions produced a growth curve similar to that obtained at 37 °C (data not shown). BoNT/A levels in culture supernatant monitored by the mouse test were identical to those measured at 37 °C (2×10^5 MLD ml⁻¹ at the end of the exponential growth phase) but lethal activity in the culture supernatant greatly decreased (2×10^2 MLD ml⁻¹) during the subsequent incubation period from 24 to 48 h. However, the transcription levels of *botR/A*, *bont/A* and *ha17* genes measured by real-time RT-PCR were of the same magnitude as those found at 37 °C (data not shown), indicating that expression of *botR/A* and the other botulinum toxin locus genes were not temperature sensitive within the 37–44 °C range. The decreased lethal activity of supernatant from 44 °C cultures probably results from post-transcriptional processing of BoNT/A.

Stability of BoNT/A and ANTPs at 44 °C

To further explore the temperature-dependent stability of BoNT/A and ANTPs, supernatant from a 37 °C Hall culture was incubated at 44 °C. As shown in Fig. 4(a), a progressive decrease in lethal activity was observed, similar to that found in a 44 °C culture during the 24–48 h incubation period. BoNT/A and ANTPs were monitored over time by Western blotting of culture supernatant incubated at 44 °C. BoNT/A significantly decreased after 0.5 h incubation at 44 °C and was not detected at 3 h or longer incubation times as monitored with antibodies specific for BoNT/A L or H chains (Fig. 4b). A similar pattern was observed with NTNH/A. After 0.5 h incubation at 44 °C, only a small amount of uncleaved NTNH/A (119 kDa) was detected, whereas the processed form (106 kDa) was not evident. In contrast, HA protein levels did not change during 24 h incubation at 44 °C (Fig. 4b). This suggests that BoNT/A and NTNH/A

Fig. 4. Proteolytic degradation of BoNT/A and NTNH/A occurs at 44 °C in Hall culture supernatant, but HA34 and HA52 (processed form of HA70) are resistant. (a) Mouse lethal activity in Hall culture supernatant obtained at 37 °C and subsequently incubated at 44 °C; (b) immunoblot analysis of neurotoxin and non-toxic components in supernatants incubated for 0, 0.5, 3, 6 and 24 h at 44 °C. Data are mean values from three independent experiments. (c) Effects of protease inhibitors on the proteolytic degradation of BoNT/A and NTNH/A at 44 °C. C. *botulinum* A strain Hall supernatant was incubated for 24 h at 37 °C (Ctrl 37 °C) or 44 °C (Ctrl 44 °C) in the absence or presence of EDTA (1 mM), PMSF (1 mM), o-phenanthroline (5 mM), benzamide (1 mM), or pepstatin (10 µg ml⁻¹). (d) BoNT/A and NTNH/A are protected from degradation in supernatant incubated for 6 h at 44 °C by either EDTA or EGTA. Lanes: 1, supernatant incubated at 37 °C; 2, supernatant incubated at 44 °C; 3–6, supernatant incubated at 44 °C with EDTA (lanes 3 and 4, 1 and 0.5 mM, respectively) or EGTA (lanes 5 and 6, 5 and 1 mM, respectively). Data are representative of three independent experiments.

were sensitive to a proteolysis at 44 °C, in contrast to HA proteins. Among the inhibitors for various proteases tested, only EDTA or EGTA (1 mM minimal concentration) blocked degradation of BoNT/A and NTNH/A (Fig. 4c, d). This indicated that a metalloprotease requiring Ca^{2+} but not Zn^{2+} , as evidenced by *o*-phenanthroline results, was active at 44 °C (not 37 °C) and degraded BoNT/A and NTNH/A in crude culture supernatant from *C. botulinum* A strain Hall.

Kinetics of BoNT/E production and relative expression of the *bont/E* and *p47* genes in *C. botulinum* E

C. botulinum type E strain HV culture at 37 °C showed a similar growth curve to type A strains, with a lag phase followed by an exponential growth and a stationary phase, although the decrease in bacterial density was slower than in *C. botulinum* A strains (Fig. 5a). The sporulation pattern was the same as for NCTC 2916 in non-sporulated cultures (data not shown). BoNT/E production was monitored in trypsin-treated culture supernatant of *C. botulinum* E strain HV grown at 37 °C. As in *C. botulinum* A strains, BoNT/E

progressively increased during the exponential growth phase and reached its maximum value at the entry into stationary phase at about 12 h (Fig. 5a). The toxicity level of the culture supernatant was then stable until at least 48 h.

Relative quantification of *bont/E* and *p47* genes was determined versus the *C. botulinum* E 16S rRNA gene during the growth of strain HV. Both genes were concomitantly expressed and their levels of transcription were similar. As found in *C. botulinum* A, expression of *bont/E* and *p47* was detectable at the beginning of exponential growth, reached a maximum level at the transition phase, and then rapidly decreased. At 24 h and longer times, RT-PCR did not detect any expression of *bont/E* and *p47* (Fig. 5b).

BoNT/E and NTNH/E are stable in culture supernatant incubated at 44 °C

We tested whether a 44 °C incubation of supernatant from a 24 h culture of strain HV at 37 °C induced a proteolytic degradation of BoNT/E and NTNH/E as found in *C. botulinum* A strain Hall. No decrease in lethal activity was

Fig. 5. Kinetics of production of botulinum neurotoxin E (BoNT/E) and relative expression of *bont/E* and *p47*. (a) Production of BoNT/E in the extracellular medium during the growth of *C. botulinum* E strain HV. The growth curve (○) was determined by measuring OD₆₀₀. BoNT/E production was assayed with the mouse test (MLD ml⁻¹, ■) using trypsinized culture supernatant. Note that BoNT/E, like BoNT/A, was detected at a low level during entry into exponential growth, increased to a maximum. (b) Relative expression of the botulinum *bont/E* (□) and *p47* (◇) versus the 16 rRNA gene during growth of *C. botulinum* E strain HV. The growth curve (○) was determined by measurement of OD₆₀₀. Data are mean values from three independent experiments. No proteolytic degradation of BoNT/E and NTNH/E occurs at 44 °C in HV culture supernatant. (c) Mouse lethal activity in HV culture supernatant obtained at 37 °C and subsequently incubated at 44 °C. (d) Immunoblot analysis of neurotoxin and non-toxic components in supernatants incubated for 0, 3, 6 and 24 h at 44 °C.

observed in the HV culture supernatant incubated at 44 °C until at least 24 h (Fig. 5c). In addition, BoNT/E and NTNH/E degradation was not evidenced by SDS-PAGE and Western blotting after incubation at 44 °C (Fig. 5d). This suggests that either no protease activated at 44 °C is produced by *C. botulinum* E or BoNT/E is more protease resistant than BoNT/A. To test this last hypothesis, we mixed equal volumes of 37 °C culture supernatant from Hall (type A) and HV (type E) strains. The mixtures were incubated at 37 °C or 44 °C for 24 h and the mouse lethal activity of BoNT/E was monitored after addition of a specific neutralizing anti-BoNT/A serum. After incubation at 37 °C, the level of mouse lethal activity was the same as in untrypsinized HV culture, indicating that BoNT/E was not activated by *C. botulinum* A proteases, in agreement with previous findings (DasGupta & Sugiyama, 1972; Dekleva & DasGupta, 1989). However, a 44 °C incubation induced a total loss of lethal activity, likely resulting from protease degradation (data not shown). This further argues that BoNT/E is sensitive to protease degradation induced by *C. botulinum* A incubated at 44 °C and that no such protease is produced by *C. botulinum* E.

DISCUSSION

The mechanism and signalling pathways controlling botulinum neurotoxin synthesis, its export into the external medium and activation by *C. botulinum* are still poorly understood. We have previously reported that BotR/A positively activates transcription of the botulinum toxin locus genes in *C. botulinum* A (Marvaud *et al.*, 1998b), acting as an RNA polymerase alternative sigma factor specific for the two promoters controlling the two operons (*ntnh/A-bont/A* and *ha-34-ha17-ha70*) of the botulinum toxin locus (Raffestin *et al.*, 2005). BotR/A is functionally related to TetR, which controls the expression of the *tet* gene in *C. tetani* *in vivo* (Marvaud *et al.*, 1998a). Moreover, BotR and TetR are also related to the alternative sigma factors TxeR and UviA, which control expression of toxin genes in *C. difficile* and a bacteriocin gene in *C. perfringens*, respectively (Dupuy *et al.*, 2005; Garnier & Cole, 1988; Mani & Dupuy, 2001). In this study, we analysed by real-time RT-PCR the relative expression of the botulinum toxin locus genes versus the 16 rRNA gene as calibrator gene (Broussolle *et al.*, 2002), in *C. botulinum* A and *C. botulinum* E strains, which in contrast to *C. botulinum* A contain no gene related to *botR*. Real-time RT-PCR is a very sensitive method to quantify gene expression and has been used to investigate various bacterial virulence genes and in particular to detect *C. botulinum* in food and clinical samples (Kimura *et al.*, 2001) and to monitor expression of neurotoxin genes in *C. botulinum* B and *C. botulinum* E (Lövenklev *et al.*, 2004a; McGrath *et al.*, 2000).

The patterns of relative expression for all botulinum toxin locus genes were identical in strains Hall and NCTC 2916 as well as in non-sporulated and sporulated cultures, although strain NCTC 2916 possesses a slightly different genetic

organization, with two botulinum toxin locus genes. One is similar to that from type A1 strains, including *ntnh*, *botR* and *has* genes, but its *bont/A* gene is replaced with a silent *bont/B* gene, while the other locus is typical of type A2 strains but has a *bont/A* type A1 gene and is organized in two operons, *p47-ntnh/A-bont/A1* and *orf1-orf2-orf3* (Dineen *et al.*, 2004; Henderson *et al.*, 1996; Jovita *et al.*, 1998). The botulinum toxin locus genes were concomitantly expressed and their kinetics showed a bell-shaped curve (Fig. 2). A low expression level was measured during entry into exponential growth, and maximal expression (50- to 200-fold increase compared to the baseline) occurred during the transition phase. Expression of the botulinum toxin locus genes then decreased to baseline at about 24 h of culture. A small increase in gene expression was observed late (48 h) in sporulated strain Hall cultures. Synchronous expression of *botR/A*, *bont/A* and *antp* genes, as well as *botR/A* being expressed about 100-fold less than the other botulinum toxin locus genes, further supports the hypothesis that BotR/A is a regulatory factor controlling *bont/A* and *antp* gene expression. In addition, the RT-PCR results clearly show that *botR/A*, *bont/A* and *antp* gene expression was mainly restricted to late exponential growth/early stationary phase, indicating that BotR/A is an alternative sigma factor specific to the transition phase and independent of sporulation. The time correlation between the expression of BotR/A and botulinum toxin locus genes suggests that BotR/A is the main regulatory factor. However, this does not rule out that another regulatory or sigma factor is also involved. These results are in agreement with a recent report showing by Northern hybridizations that *bontA*, *ntnhA* and *ha* genes are temporally expressed during late exponential and early stationary phase in *C. botulinum* type A strains 62A, Hall A-hyper and NCTC 2916 (Bradshaw *et al.*, 2004). It is noteworthy that TxeR from *C. difficile* is also predominantly expressed during the transition phase, as evidenced by a fusion between a β -glucuronidase reporter gene and TxeR in *C. perfringens* (Karlsson *et al.*, 2003).

Analysis of mRNA by Northern blotting and transcriptional study with purified BotR/A-RNA polymerase core enzyme has indicated that the *bont/A* and *antp* genes from *C. botulinum* type A1 strains such as Hall strain are transcribed in two divergent operons (*ntnh/A-bont/A1* and *ha34-ha17-ha70*) and that *bont/A1* can also be expressed separately (Bradshaw *et al.*, 2004; Henderson *et al.*, 1996; Raffestin *et al.*, 2005). RT-PCR results showing equivalent expression levels of each gene of the botulinum toxin locus in the Hall strain are in agreement with the genetic organization in two operons. However, strain NCTC 2916 yielded twofold more expression of *ha34* versus *ha17* and *ha70*. It is noteworthy that relative amounts of HA proteins vary in the different-sized botulinum complexes. Indeed, the ratio of HA34 versus HA17 or HA70 is about twice in 19S compared to 12S complexes (Inoue *et al.*, 1996; Oguma *et al.*, 1999; Sharma *et al.*, 2003). Regulation of HA production at the transcription and/or translation level in *C. botulinum* strains such as in NCTC 2916 remains to be defined.

BoNT/A was detected in the culture supernatant at low levels during early exponential growth and reached a maximum level at 24 h of culture, which remained stable until at least 72 h. No obvious bacterial lysis was observed during the transition and early stationary phases in either strain. This indicates that BoNT/A is essentially synthesized during the transition into early stationary phase and is transported in a delayed manner into the external medium. Similar patterns of growth and toxin production without apparent cell lysis were previously reported (Bradshaw *et al.*, 2004; Siegel & Metzger, 1979). An exfoliation process without cell lysis has been reported (Call *et al.*, 1995). It could be argued that BoNT degradation and resynthesis take place during stationary phase; however, BoNT/A is activated by an endogenous protease from *C. botulinum* and is stable in acidic cultures at 37 °C (DasGupta, 1989).

An important question is which are the environmental factors controlling neurotoxin gene production in *C. botulinum*. Although nitrogen and carbon sources are important for the toxinogenesis, specific external signals controlling toxinogenesis have not been clearly identified in *C. botulinum* or *C. tetani*. In contrast to *C. difficile* (Dupuy & Sonenshein, 1998), *C. botulinum* requires glucose for high toxin production. CO₂ seems to stimulate *bont/B* gene expression and subsequent neurotoxin production in *C. botulinum* type B (Lövenklev *et al.*, 2004b). Moreover, short peptides from media containing pancreatic digest of casein are essential for toxin production by *C. tetani* (Porfirio *et al.*, 1997). Growth temperature is a common signal involved in the control of alternative sigma factors and subsequent regulated gene expression. Thereby a temperature of 42 °C prevents transcription of *txeR* and the subsequent expression of *toxA* and *toxB* genes in *C. difficile* (Karlsson *et al.*, 2003). Cultures of *C. botulinum* Hall incubated at 44 °C showed an initial toxin production similar to that found in cultures at 37 °C and then a dramatic decrease in toxin activity after 24–48 h of growth (data not shown), which is in agreement with previous observations (Siegel & Metzger, 1979). However, no decrease in *botR/A*, *bont/A* and *antp* gene expression was observed in cultures at 44 versus 37 °C, indicating that declining toxin activity resulted not from gene regulation but rather from a post-translational event. Our data show that a calcium-dependent protease degraded BoNT/A in culture supernatant incubated at 44 °C. Interestingly, the same type of protease is responsible for BoNT/A activation into the di-chain form (Dekleva & DasGupta, 1990). Characteristics of the BoNT/A-activating protease from *C. botulinum* A include: (1) a thiol-reducing agent and Ca²⁺ requirement; (2) maximum activity in the pH range 6.2–6.4; and (3) a temperature optimum of 70 °C (Dekleva & DasGupta, 1990). Although weakly active at 37 °C, this protease efficiently activates BoNT/A by a single proteolytic nicking and excision of 10 residues between the light and heavy chains (Krieglstein *et al.*, 1994), whereas increased activity at 44 °C caused neurotoxin degradation (Fig. 4) by cleaving multiple sites. This is further supported by the fact that prolonged exposure of BoNT/A to purified protease led

to considerable toxin degradation in multiple fragments (Dekleva & DasGupta, 1990). Seemingly, *C. botulinum* A uses a temperature-dependent protease to control BoNT/A activation and degradation. We can not rule out that an increase in temperature also induces a conformational change in BoNT/A, leading to exposure of cleavage sites on the surface of the molecule. One of the functions for ANTPs may be to protect BoNT/A against protease degradation. As shown in Fig. 4, NTN/A was as sensitive as BoNT/A to proteolytic degradation at 44 °C, whereas HA34 and HA52 were resistant. Trypsin or pepsin resistance of HA34 versus NTN/A at 25 °C has already been observed (Chen *et al.*, 1998; Fu *et al.*, 1997; Sharma *et al.*, 1999). Since BoNT/A from culture supernatant with increased temperature was degraded, it is excluded that HA proteins have a protective effect for the neurotoxin, at least against this type of protease.

In contrast to the other *C. botulinum* types, *C. botulinum* E strains do not contain an equivalent of the *botR* gene in their botulinum toxin locus, and no related gene has been identified in their genome (Quinn & Minton, 2001). However, *p47*, which encodes a 47 kDa protein, is located immediately upstream of *ntnh/E* and *bont/E*, similarly to *botR/A* in *C. botulinum* A (Popoff & Marvaud, 1999). In contrast to *botR/A*, *p47* was expressed at a level similar to that of *bont/E*. This is further supported by the fact that *C. botulinum* E transformed with a recombinant plasmid expressing *p47* antisense mRNA did not decrease BoNT/E synthesis (data not shown), in contrast to *botR/A* antisense mRNA in *C. botulinum* A (Marvaud *et al.*, 1998b), indicating that P47 is probably not a regulator of the botulinum toxin E locus genes. Interestingly, *bont/E* and *p47* were selectively expressed at the transition phase, as the botulinum toxin A locus genes, but by an as yet unknown regulator.

A possible regulation of the toxinogenesis in *C. botulinum* E by high temperature was investigated. However, *C. botulinum* E strains did not grow at 44 °C and only weakly at 40 °C. This group of bacteria is adapted to grow at low temperature and the optimum temperature is around 25–30 °C (Hatheway, 1989). Toxinogenesis was the same at 37 versus 30 °C (data not shown), suggesting that high temperature is probably not involved in toxin gene regulation by *C. botulinum* E. *C. botulinum* E strains are non-proteolytic and trypsin treatment of the culture supernatant is required to obtain fully active BoNT/E (Hatheway, 1989). In contrast to *C. botulinum* A strain Hall, no decrease in BoNT/E activity and no proteolytic degradation of BoNT/E and NTN/A were apparent in culture supernatant incubated at 44 or 37 °C. This argues that a unique protease is involved in activation/degradation of BoNTs in proteolytic *C. botulinum* strains such as *C. botulinum* A.

In conclusion, the *botR/A*, *bont/A* and *antp* genes of *C. botulinum* A are mainly expressed during the transition from exponential into stationary phase, the level of *botR/A* expression being about 100-fold less than that for the *bont/A*

and *antp* genes. This further argues that BotR/A is an alternative sigma factor controlling botulinum toxin A locus genes during the transition phase. In *C. botulinum* E, expression of *bont/E* and *p47* is also positively regulated to an equivalent extent during the transition phase but by an unknown factor. Although high temperature is not an inhibitory signal for toxin gene regulation, it stimulates a calcium-dependent protease, which activates BoNT/A at 37 °C but degrades BoNT/A and NTNH/A at 44 °C. No such activating or degradative protease was found in *C. botulinum* E.

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