Real-time PCR for diagnosis of botulism and quantification of neurotoxin gene expression in Clostridium botulinum

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Real-time PCR for diagnosis of botulism and quantification of neurotoxin gene expression in *Clostridium botulinum*

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Abstract

The objectives of the work presented in this thesis have been to develop molecular methods for (i) detection of *Clostridium botulinum* in food and clinical samples, and (ii) monitoring the expression of the neurotoxin gene, *cnt*, during different growth phases and under different growth conditions.

For the diagnosis of botulism, a real-time PCR-based method including an internal amplification control for *C. botulinum* types A, B and E was developed. The method was used to diagnose the first case of wound botulism in an injecting drug user in Sweden, which was also the first case of wound botulism reported to be caused by *C. botulinum* type E. It was previously assumed that only proteolytic *C. botulinum* could cause infectious botulism. However, this case revealed that wound botulism can be caused by nonproteolytic *C. botulinum*.

When developing new food products it is important to consider not only the occurrence and quantity of pathogens in the food chain, but how the processing and subsequent storage and handling will affect them. Especially for food designed to have a long shelf-life, understanding the influence of environmental factors, food preservatives, and type of packaging on microbial growth and virulence expression is very important.

Quantitative reverse transcription PCR (qRT-PCR) and enzyme-linked immunosorbent assay (ELISA) were used to monitor the neurotoxin expression in *C. botulinum* types A, B, and E. The relative *cnt* expression varied with growth phase in all three types, reaching a maximum as the late exponential phase was changing into stationary phase. However, for the proteolytic type A, a second increase could be seen in late stationary phase in contrast to type E. The same behaviour has earlier been observed for proteolytic *C. botulinum* type B with a second increase in *cntB* mRNA when the bacteria reached the death phase. For the nonproteolytic *C. botulinum* type E the *cntE* expression declined rapidly after the peak in the transition between exponential and stationary phase. The *cntE* mRNA half-life was calculated to be approximately nine minutes.

When investigating the effect of carbon dioxide on growth and neurotoxin expression, we found that while proteolytic type A was not affected in either regard, the nonproteolytic types B and E were. Their growth was slowed, but the relative *cnt* expression was increased with an elevated CO_2 concentration, with fivefold higher levels of *cntB* mRNA and type B toxin being detected at 70% CO_2 than at 10%. For type E the increase in expression at 70% CO_2 was two-fold greater, when compared with 10% CO_2. For the proteolytic strain Hall A microarrays

were also used to follow the genome-wide expression profiles. Comparison between the results from the qRT-PCR and the microarrays shows that both methods seem valid, as the results are similar. For example, the expression of the positive regulator gene, *cntR,* followed the same pattern as *cntA*, but at a much lower level.

In conclusion, our findings, confirmed with both qRT-PCR and ELISA, shed a new cautionary light on the potential risks of botulism associated with nonproteolytic *C. botulinum* and the use of modified atmosphere packaging. In addition, our PCR method was successfully used on clinical samples and proved to be a valuable complement to standard methods.

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List of papers

This thesis is based on the following papers, referred to in the text by their Roman numerals.

Paper I

<u>Artin, I.</u>, P. Björkman, J. Cronqvist, P. Rådström and E. Holst (2007).
First Case of Type E Wound Botulism Diagnosed Using Real-time PCR.
Journal of Clinical Microbiology **45**(11): 3589-94.

Paper II

Lövenklev, M., <u>I. Artin</u>, O. Hagberg, E. Borch, E. Holst and P. Rådström (2004).
Quantitative Interaction Effects of Carbon Dioxide, Sodium Chloride, and Sodium Nitrite on Neurotoxin Gene Expression in Nonproteolytic *Clostridium botulinum* Type B.
Applied and Environmental Microbiology 70(5): 2928-34.

Paper III

<u>Artin, I.</u>, A. T. Carter, E. Holst, M. Lövenklev, D. R. Mason, M. W. Peck, and P. Rådström.
Effects of Carbon Dioxide on Neurotoxin Gene Expression in Non-Proteolytic *Clostridium botulinum* Type E.
Applied and Environmental Microbiology. Submitted for publication.

Paper IV

<u>Artin, I.</u>, D.R. Mason, J. Schelin, M.W. Peck, E. Holst, P. Rådström, and A.T. Carter.
Effect of Carbon Dioxide on Growth and the Transcriptome of Proteolytic *Clostridium botulinum* Type A.
Manuscript.

Abbreviations

ANTP   associated non-toxic proteins

ASM   American Society for Microbiology

BoNT   botulinum neurotoxin

cDNA   complementary DNA

CFS   cerebrospinal fluid

cnt   botulinum neurotoxin gene

cntR   regulatory protein gene

CO_2 carbon dioxide

Ct   threshold cycle

ECL   electrochemiluminescence

ELCA   enzyme-linked coagulation assay

ELISA   enzyme-linked immunosorbent assay

EMG   electromyography

FDA   US Food and Drug Administration

FRET   fluorescence resonance energy transfer

GMO   genetically modified organisms

HA   hemagglutinating proteins

ha   hemagglutinating protein gene

HC   heavy chain

IAC   internal amplification control

KH   Henry's law's constant

LC   light chain

MAP   modified atmosphere packaging

mRNA   messenger ribonucleic acid

MS   mass spectrometry

N_2 nitrogen

NSF   N-ethylmaleimide-sensitive fusion protein

NTNH   non-toxic non-hemagglutinating proteins

ntnh   non-toxic non-hemagglutinating protein gene

O_2 oxygen

OD   optical density

ORF   open reading frame

p47	47 kDa protein in neurotoxin cluster
pCO_2	partial pressure of CO_2
PCR	polymerase chain reaction
qRT-PCR	quantitative RT-PCR
RT-PCR	reverse transcription PCR
SNAP-25	synaptosomal-associated protein of 25 kDa
SNARE	soluble NSF-attachment protein receptor
VAMP	vesicle-associated membrane protein

Clostridium botulinum

The organism

In 1820 Justinius Kerner described an outbreak where 76 people suffered from descending flaccid paralysis after eating smoked sausage. He called the disease "botulism" after the Latin word for sausage, *botulus*. Two years later he published a monograph on the "sausage poison" which contained a clinical evaluation and summary of 155 cases, including post-mortems, as well as a description of his experiments in purifying the toxin and administering it to animals. He deduced that the toxin worked by interrupting signal transmission in the muscles but not the sensory signal transmission, comparing it to rust in an electrical conductor. He even went so far as to test the toxin on himself (Erbguth and Naumann, 1999).

In 1895 Emile van Ermengem isolated a bacteria from salted ham that had caused an epidemic, with the symptoms described by Kerner, in Ellezelles (van Ermengem, 1979). What he found was gram-positive anaerobic spore-forming slightly curved rods with oval subterminal spores that he named *Bacillus botulinus*. The organism was later renamed *Clostridium botulinum*. Furthermore, he found that the disease was not caused directly by the bacteria themselves, but by a heat-labile toxin.

Later studies have shown that *C. botulinum* are motile bacteria with peritrichous flagellae and that their size varies in the range 0.3-0.7 × 3.4-7.5 μm. *C. botulinum* exhibits great cultural, physiological, and genetic diversity. Today these bacteria are known to produce seven types of botulinum neurotoxins (BoNTs), based on the serological specificity of their toxins, designated A-G. Four phenotypic groups (I-IV) can be found that differ so much that they could be considered different species (Suen et al., 1988b). Group I strains producing BoNT types A, B, and F are proteolytic and mesophilic with an optimal growth temperature of 35-40°C and a minimum growth temperature of 10°C. The spores are highly heat-resistant (Hatheway, 1993). Group II strains producing toxin types B, E, and F are nonproteolytic psychrotrophs with an optimal growth temperature of 30°C or below and a minimum growth temperature of 3°C (Graham et al., 1997). The spores have low heat resistance and, since the cells lack endogenous proteolytic enzymes for toxin activation, the toxicity can be increased by trypsinisation (Hatheway, 1993).

For both group I and II the toxin gene is generally considered to be chromosomal, though recently two strains have been found where the toxin genes were located in a plasmid (Marshall et al., 2007). Group III consists of BoNT types C and D, and they are generally

nonproteolytic. They have an optimal growth temperature of 40°C and a minimum growth temperature of 15°C. The spores have an intermediate heat resistance and the toxicity gene is phage-mediated (Eklund and Poysky, 1974). Group IV consists of type G strains and are proteolytic, asaccharolytic, and lipase-negative, have an optimal growth temperature of 37°C and spores with an intermediate heat resistance. The toxicity is enhanced by trypsinisation (Hatheway, 1993). The BoNT gene is located on a plasmid (Eklund et al., 1988; Zhou et al., 1995).

Group I and II are known to cause botulism in humans, usually from BoNT types A, B, and E, while group III (types C and D) are considered animal pathogens. Group IV (type G) has not been associated with disease, but has been found in necropsy of humans who died suddenly of an unidentified cause (Schiavo et al., 1994) and in cases reported as sudden infant death (Sonnabend et al., 1985).

The standard definition of *C. botulinum* includes all organisms known to produce BoNTs and thereby able to cause botulism in humans or animals (Prévot, 1953). However, strains have been found that are phenotypically and genetically *C. butyricum* and *C. baratii* that produce type E and F toxin respectively (Suen et al., 1988b), and *C. botulinum* type G is sometimes considered to be a different species, *C. argentinense* (Suen et al., 1988a).

C. botulinum is considered to be a ubiquitous soil bacterium, but the type of spores present varies between locations and soil type. In the western United States, especially west of the Rocky Mountains, mainly type A spores are found, generally in neutral to alkaline soil with a low organic content. In the eastern United States type B spores predominate, mainly in slightly acidic soil with a higher organic content. Type E is mainly found in connection to water, e.g. in the Great Lakes area, in shore soil, and sediment. In inland soil in Europe type B spores dominate, except in Sweden, where mainly type E is found (Dodds, 1993). The variations between different locations and types of food can be seen in Table 1, where some recent cases of foodborne outbreaks of botulism are summarised.

Table 1. Recent cases of foodborne outbreaks of botulism

Year	Country	Food implicated	Cases	Toxin type*	Reference
1989	UK	Hazelnut purée added to yoghurt	27	B	(Brett, 1999)
1989	USA	Garlic-in-oil	3	A	(Morse et al., 1990)
1991	Sweden	Vacuum-packed hot-smoked rainbow trout	2	E	(Anonymous, 1991)
1992	USA	Uneviscerated salt-cured fish product (moloha)	4	E	(CDC, 1992)
1993	Italy	Roasted eggplant in oil	7	B	(CDC, 1995b)
1993	USA	Commercial canned cheese sauce	8	A	(Townes et al., 1996)
1994	USA	Baked potato	30	A	(Angulo et al., 1998)
1994	USA	Beef stew	1	A	(CDC, 1995a)
1994	Sweden	Hot-smoked salmon	3	nd	(Öberg, 1994)
1994	Sweden	Home-preserved fish	1	nd	(Öberg, 1994)
1994	China	Salted, fermented paste of soybeans and wax gourds	6	*C. butyricum* / E	(Meng et al., 1997)
1995	Italy	Canned macrobiotic food	1	Ab	(Franciosa et al., 1997)
1995	Canada	Seal oil	1	E	(Proulx et al., 1997)
1995	Canada	Fermented seal meat	3	E	(Proulx et al., 1997)
1995	Canada	Fermented walrus meat	5	E	(Proulx et al., 1997)
1995	Canada	Fermented seal meat	1	E	(Proulx et al., 1997)
1996	Italy	Mascarpone cream cheese	8	A	(Aureli et al., 2000)
1996	India	Sevu	34	*C. butyricum* / E	(Chaudhry et al., 1998)
1996	USA	Home-preserved tomato sauce with meat	1	B	(Shapiro et al., 1997)

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Table 1. *Continued*

Year	Country	Food implicated	Cases	Toxin type	Reference
1997	USA	Home-pickled eggs	1	B	(CDC, 2000)
1997	Finland	Vacuum-packed hot-smoked whitefish	2	E	(Korkeala et al., 1998)
1997	Norway	Rakfisk	4	E	(Kuusi et al., 1999)
1997	Argentina	Home-cured ham	6	E	(Rosetti et al., 1999)
1997	Iran	Locally made cheese	27	A	(Pourshafie et al., 1998)
1998	UK	Bottled mushrooms	2	B	(Brett, 1999)
1998	Thailand	Home-canned bamboo shoots	13	A	(CDC, 1999)
1998	Denmark	Commercially produced vegetable pie	1	B	(Poulstrup et al., 1999)
1999	Finland	Whitefish eggs	1	E	(Lindström et al., 2004)
2000	France	Home-canned asparagus	9	B	(Abgueguen et al., 2003)
2001	USA	Fermented beaver	7	E	(CDC, 2001)
2001	Canada	Fermented salmon roe	2	E	(Dawar et al., 2002)
2001	Canada	Fermented salmon roe	2	E	(Dawar et al., 2002)
2001	USA	Canned tuna / Spagetti noodles and meat sauce [a]	1	*C. baratii* / F	(Harvey et al., 2002)
2001	USA	Frozen chili	16	A	(Kalluri et al., 2003)
2002	USA	Muktuk from beached whale	8	E	(CDC, 2003)
2002	South Africa	Tinned fish in tomato sauce	2	A	(Frean et al., 2004)
2003	Denmark	Garlic in chili oil	1	B	(Lohse et al., 2003)
2003	UK / Poland	Sausage	2	B	(McLauchlin et al., 2006)

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Table 1. *Continued*

Year	Country	Food implicated	Cases	Toxin type	Reference
2004	UK	Hummus	1	nd	(McLauchlin et al., 2006)
2005	Turkey	Roasted canned mushrooms	5	nd	(Cengiz et al., 2006)
2005	UK	Home-preserved pork	1	B	(McLauchlin et al., 2006)
2005	USA	Home-salted fish	5	E	(Sobel et al., 2007)
2006	Thailand	Home-canned bamboo shoots	163	A	(CDC, 2006b)
2006	USA	Commercial carrot juice	4	A	(CDC, 2006a)
2006	USA	Home-prepared fermented tofu	2	A	(CDC, 2007b)
2006	Finland	Vacuum-packed smoked whitefish	1	E	(Lindström et al., 2006b)
2006	Austria	Barbecued pork [b]	5	nd	(Meusburger et al., 2006)
2007	USA	Commercially canned chili sauce	5	A	(CDC, 2007a)

* *C. butyricum* / E – Type E toxin produced by *C. butyricum.*

C. baratii / F – Type F toxin produced by *C. baratii.*

nd – Toxin type not determined.

[a] Toxin found in both, not certain if this was true at time of consumption.

[b] No *C. botulinum* or toxin could be isolated

.

The toxin

Botulinum toxins are produced in complexes with associated non-toxic proteins (ANTP). These can include non-toxic non-hemagglutinating proteins (NTNH) and hemagglutinating components (HA). The size of the complexes varies between 300 and 900 kDa, depending on both intrinsic and extrinsic factors (Johnson and Bradshaw, 2001). Their function is not clear, but probably relate to protecting the toxin and helping it stay active after ingestion. The BoNTs are zinc endopeptidases (Schiavo et al., 1992), which share the amino acid sequence with the consensus sequence common to other Zn^{2+}-dependent metallopeptidases (Ahnert-Hilger and Bigalke, 1995). All the BoNTs bind one zinc atom per molecule, except BoNT/C, which binds two (Humeau et al., 2000). They are synthesised as single-chain 150 kDa polypeptides, and then cleaved by proteolytic nicking within a surface-exposed loop at the LC-H_N junction by bacterial or tissue proteases to give the active toxin.

The di-chain molecule consists of a light chain (LC) pharmacodynamic part of 50 kDa and a heavy chain (HC) pharmacokinetic part of 100 kDa that are linked by a disulfide bond (Ahnert-Hilger et al., 1995). The heavy chain consists of two 50 kDa domains. The C-terminal domain of the HC (H_C) is the ganglioside-binding domain. It binds to target cell membrane and internalises the toxin molecules. The binding is highly selective, binding only to gangliosides with more than one neuraminic acid residue, which is only found in high concentrations in nerve cell membranes. The toxin is internalised by the nerve cell while forming a ternary complex with the ganglioside and the receptor protein. Internalisation takes place by receptor-mediated endocytosis involving clathrin-coated vesicles (Ahnert-Hilger et al., 1995). H_N is the translocation domain, which allows the LC to escape from the endosomal compartment into the cytosol (Bade et al., 2004). It forms ion channels in lipid bilayers and is most active at endosomal pH.

The H_N domain has a pair of long (105 Å) amphipathic helices, resembling coiled coils of viral proteins, and a "translocation belt", a long loop wrapped around the LC (Turton et al., 2002). In this way the H_N masks the catalytic cleft of the LC when the disulfide bridge is intact (Humeau et al., 2000), possibly to protect it from auto-cleavage (Ahmed et al., 2001). A comparison between the loop of BoNT/A and SNAP-25 shows both spatial and sequence homology, suggesting a mimicking behaviour between the loop and the initial recognition of SNAP-25 (Chen et al., 2007). The light chain is the zinc-dependent endopeptidase and contains the catalytic zinc atom and a conserved HExxH motif. The catalytic site, with negative surface charge, is located in a deep cleft (20 Å), which is accessible through a

channel. In order for the LC to pass through the channels formed by the HC there needs to be an acid pH in the *cis* side, like in the endosome, and a reducing environment in the *trans* side, like in the cytosol (Koriazova and Montal, 2003). It is thought that the LC partially unfolds forming a molten globule structure (Bade et al., 2004) at endosomal pH in order to pass through the HC channel, which has a diameter (~15 Å) smaller than the folded LC (55 Å × 55 Å × 62 Å) (Lacy et al., 1998). When it has passed through to the cytosol, it refolds at the interface and dissociates from the HC (Koriazova et al., 2003). This reduction of the disulfide bond is necessary for intracellular action during intoxication (Humeau et al., 2000).

Once inside the cytosol the protease can start cleaving one of the pre-synaptic proteins. These are SNAP-25 (synaptosomal-associated protein of 25 kDa), synaptobrevin (or VAMP – vesicle-associated membrane protein), and syntaxin. These are collectively referred to as SNAREs and form a synaptic fusion complex, which is a heterotrimeric, parallel four-helix bundle, that mediates the fusion of a vesicle to its target membrane (Sutton et al., 1998). Only uncomplexed or partially assembled SNARE proteins can be cleaved by the neurotoxin.

SNAP-25 is specifically cleaved by BoNT/A and BoNT/E at two distinct carboxyl terminal sites. BoNT/A cleaves Gln^{197}-Arg^{198} and BoNT/E cleaves Arg^{180}-Ile^{181} (Schiavo et al., 1993a). Synaptobrevin is cleaved by BoNT/B at Gln^{76}-Phe^{77}(Schiavo et al., 1992), BoNT/D at Lys^{59}-Leu^{60}(Yamasaki et al., 1994), BoNT/F at Gln^{58}- Lys^{59} (Schiavo et al., 1993b), and by BoNT/G at Ala^{81}-Ala^{82} (Schiavo et al., 1994). BoNT/C cleaves syntaxin at Lys^{253}-Ala^{254} (Schiavo et al., 1995) and can also cleave SNAP-25 (Humeau et al., 2000). The mechanism of action can be seen in Figure 1.

For a human weighing 70 kg the lethal dose of crystalline type A toxin has been estimated to be 0.09-0.15 µg intravenously or intramuscularly, 0.7-0.9 µg inhalationally and 70 µg orally (Arnon et al., 2001).
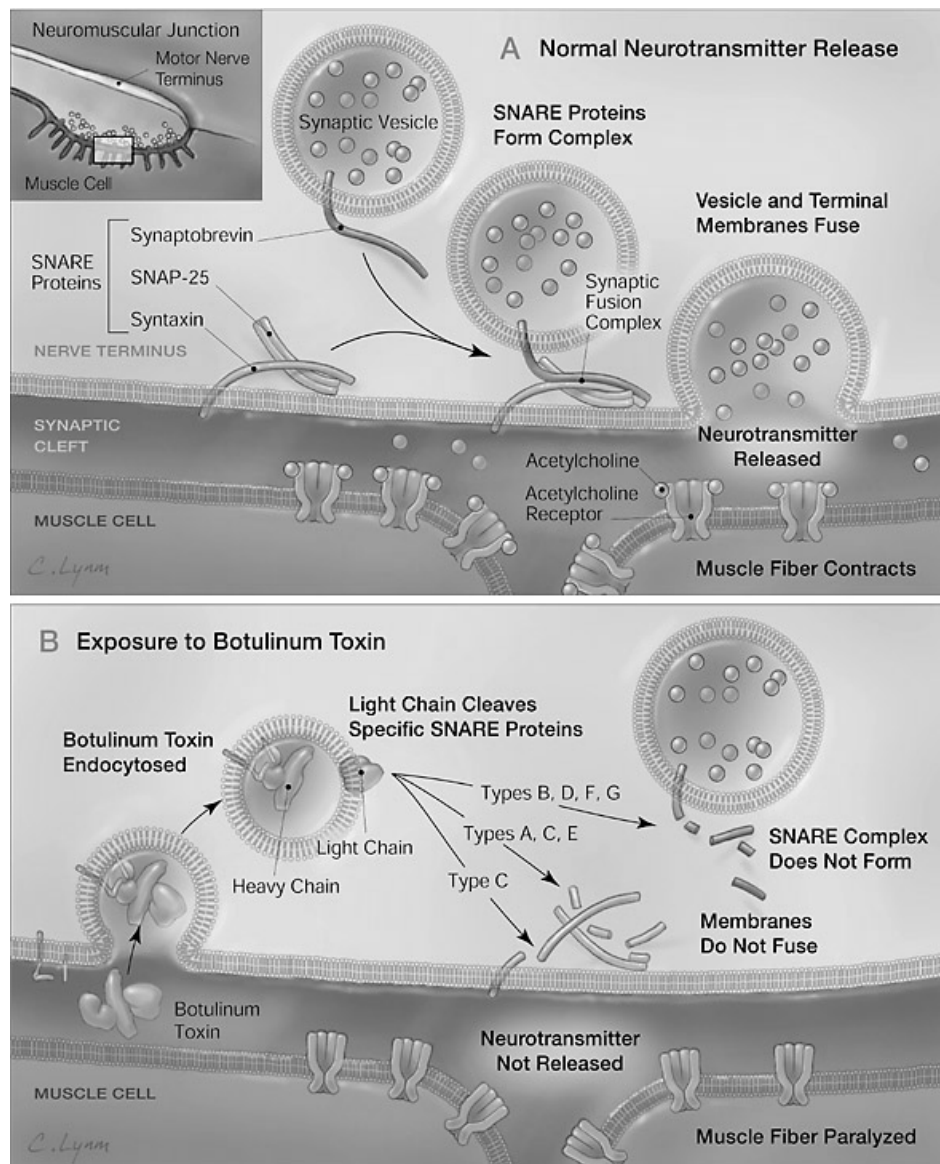
Figure 1 Mechanism of action of botulinum toxin (Arnon et al., 2001). Copyright JAMA.

A. Release of acetylcholine at the neuromuscular junction is mediated by the assembly of a synaptic fusion complex that allows the membrane of the synaptic vesicle containing acetylcholine to fuse with the neuronal cell membrane. The synaptic fusion complex is a set of SNARE proteins, which include synaptobrevin, SNAP-25, and syntaxin. After membrane fusion, acetylcholine is released into the synaptic cleft and then bound by receptors on the muscle cell.

B. Botulinum toxin binds to the neuronal cell membrane at the nerve terminus and enters the neuron by endocytosis. The light chain of botulinum toxin cleaves specific sites on the SNARE proteins, preventing complete assembly of the synaptic fusion complex and thereby blocking acetylcholine release. Botulinum toxins types B, D, F, and G cleave synaptobrevin; types A, C, and E cleave SNAP-25; and type C cleaves syntaxin. Without acetylcholine release, the muscle is unable to contract. SNARE indicates soluble NSF-attachment protein receptor; NSF, N-ethylmaleimide-sensitive fusion protein; and SNAP-25, synaptosomal-associated protein of 25 kDa.

The disease

Botulism can be divided into several different categories; classic or foodborne botulism, wound botulism, infant botulism, adult infectious botulism, inadvertent botulism and inhalation botulism.

Foodborne botulism is an intoxication caused by consuming food containing preformed neurotoxin. It is the most common cause of botulism in Europe. Many different types of food have been implicated, but the most common ones are home-made preserved foods. The type of toxin varies with location and type of food. For fish and seafood, type E is the most common, while in meats and vegetables types A and B dominate (Dodds, 1993a). The incubation period is usually 18-36 hours (Bell and Kyriakides, 2000). The first symptoms are often gastrointestinal, e.g. nausea, vomiting, stomach cramps, diarrhoea, or constipation. Many patients complain about having a dry mouth, muscle weakness and blurred or double vision (diplopia). Other symptoms are dysphagia (difficulty in swallowing), dysphonia (difficulty in speaking), dysarthria (slurred speech), ptosis (drooping eyelids), ophthalmoplegia (eye muscle paralysis), and a symmetric descending paralysis. It begins with the cranial nerves, followed by the upper extremities, the respiratory muscles, and the lower extremities (Shapiro et al., 1998).

Wound botulism occurs when a wound is contaminated with *C. botulinum* spores which germinate and produce toxin *in situ*. Wound botulism has been, except at times of war, a very rare form of botulism. It occurred mainly in patients with traumatic and surgical wounds, contaminated with dirt, e.g. shrapnel wounds. Between 1943 and 1990 only 47 cases were reported to the CDC and in literature (Cherington, 1998). Since then, however, there has been an upsurge of cases (Cherington, 1998; Jensen et al., 1998; Passaro et al., 1998; Jensenius et al., 2000; Brett et al., 2004; Akbulut et al., 2005; Alpers et al., 2005) (Paper I). The new risk group is injecting drug users, especially if they use skin or muscle "popping". Soft tissue infections at injection sites can be caused by several different *Clostridium spp.* as well as by *Bacillus cereus* (Mitchell and Pons, 2001; Dancer et al., 2002). This may be due to dirty injecting paraphernalia or contaminated drugs. Drugs are often dissolved in acids and this enhances the breakdown of tissues and may accelerate bacterial growth. The symptoms are similar to those in classical botulism, but without gastrointestinal symptoms, and with an incubation period of 4-14 days (Shapiro et al., 1998). The symptoms may also appear in a different order than the classical picture (Brett et al., 2004).

Infant botulism occurs when *C. botulinum* colonises the intestines of infants younger than 12 months and produces toxin *in situ*. It is the most common cause of botulism in the United States, where 90% of the world's cases are diagnosed (Cox and Hinkle, 2002). It is believed that colonisation can occur since a normal bowel flora has not yet been established that could compete with *C. botulinum* (Sobel, 2005). The symptoms include constipation, lethargy, listlessness, general muscle weakness and "floppy head", weak cry, poor sucking ability and difficulty in swallowing (Midura, 1996). There is a risk that the fulminant form, which leads to sudden and unexpected death, is mistaken for sudden infant death (Sonnabend et al., 1985; Bohnel et al., 2001; Nevas et al., 2005). Soil and honey are the two recognised sources of spores involved in infant botulism, but at least in one case infant formula milk powder has been implicated (Brett et al., 2005).

Adult infectious botulism, sometimes called hidden botulism, is similar to infant botulism in that the intestinal tract is colonised and the toxin produced *in situ*. It is unusual and occurs mainly in patients with an abnormality in the gastrointestinal tract, e.g. prior surgery, achlorhydria, Crohn's disease, or recent antibiotic treatment (Cherington, 1998).

It has long been held that the infectious forms of botulism (wound, infant, and hidden) can only be caused by type I strains, *C. butyricum*, and *C. baratii*, which all have optimal growth temperatures close to body temperature (Dodds, 1993c). However, we diagnosed a case of wound botulism caused by *C. botulinum* type E, a type II strain (Paper I).

Inadvertent, or iatrogenic, botulism has been caused by injection of botulinum toxin for cosmetic or therapeutic purposes. It seems to have occurred both in patients treated with doses below the maximum recommended dose (Cherington, 1998) and when unlicensed, highly concentrated toxin was used (Sobel, 2005; Chertow et al., 2006).

Inhalation botulism may result from aerosolisation of neurotoxin (Lindström and Korkeala, 2006a) and is not a naturally occurring disease (Sobel, 2005). It may occur in the laboratory setting when enough care is not taken or through the deliberate dissemination of the toxin (Arnon et al., 2001), e.g. as a weapon of bioterrorism.

Treatment of botulism consists mainly of advanced medical and nursing supportive care (Cherington, 1998). The mortality rate dropped sharply when mechanical ventilation was introduced in the 1940s and 1950s (Sobel, 2005). The only specific treatment for botulism is botulinum antitoxin. It can stop the progression of paralysis and decrease the time of hospitalisation. To give the best effect it should be administered early, preferable within 24 h of the onset of symptoms, since it only neutralises unbound toxin molecules (Sobel, 2005). However, effect has been seen as late as after four days (Chang and Ganguly, 2003). Since

this antitoxin is of equine origin there is a problem with adverse effects, including allergic reactions, anaphylactic shock, and serum sickness. This means that skin testing should be done before administration and that it can not be given to infants.

For infants, only supportive and respiratory care is given. The prognosis is still good, with a fatal outcome in less than 2% of the cases. Antibiotic treatment should not be given, as this may cause toxin to be released when the cells die and lyse. It may also destroy the normal microbial flora, helping overgrowth of *C. botulinum*. Some antibiotics, like the aminoglycosides, can even increase the neuromuscular weakness caused by *C. botulinum*, e.g. gentamicin may potentiate sublethal concentrations of toxin, resulting in complete neuromuscular blockade (Brook, 2007).

Recently an antitoxin of human immunoglobulin has been tested for the treatment of infant botulism (Arnon et al., 2006). The results seem promising, with a reduction in the length of hospital stay, but so far this antitoxin is only available in the US. Work is also being done to develop recombinant monoclonal antibodies as antitoxin, but this work is complicated by the existence of several subtypes within the serotypes (Smith et al., 2005). In the case of wound botulism suspected sites of infection are surgically debrided and intravenous benzylpenicillin combined with metronidazole given as well as antitoxin (Sieradzan, 2005).

Genetics

The *C. botulinum* genome has a very low content of d(G/C) DNA. The preferred initiation and termination sequences are AUG and UAA, and the use of degenerate codons ending in C or G is discriminated against. In the gene encoding neurotoxin type A, *cntA*, 90.3% of degenerate codons end in A or U (Thompson et al., 1990).
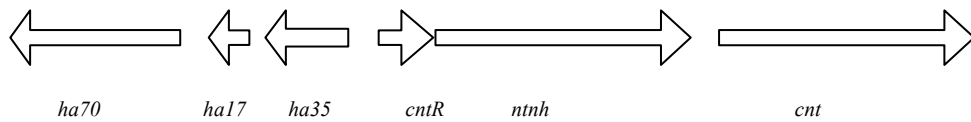
The nucleotide sequence of the neurotoxin gene has been identified for all toxin types, as well as some of the surrounding genes (Binz et al., 1990a; Binz et al., 1990b; Kimura et al., 1990; Thompson et al., 1990; East et al., 1992; Poulet et al., 1992; Whelan et al., 1992; Campbell et al., 1993). Recently the complete genome of three *C. botulinu*m type A strains and one type F strain have been reported (accession CP000726, CP000727, AM412317, and CP000728), as well as the sequence of the plasmid pBOT3502 in ATCC 3502 (AM412318) and the type C neurotoxin-converting phage (AP008983).

The neurotoxin genes (*cnt*) in *C. botulinum* are located in a cluster with the genes coding for the ANTPs in the botulinum locus, and all the genes in the cluster are expressed together (Bradshaw et al., 2004; Couesnon et al., 2006) (Paper IV). The genes are transcribed as two divergent polycistronic messages, with the *ntnh* and *cnt* in one and *ha70*, *ha17* and *ha33* in the other. The organisation of the genes in the locus varies, as can be seen by the variation in the neurotoxin complex size. The 3' part tends to be more conserved than the 5' part (Raffestin et al., 2004). A gene called *botR* or *cntR*, also located in the locus, has been found in all types except non-proteolytic type E and acts as a positive regulator (Marvaud et al., 1998). The organisation of the genes in the different types can be seen in Figure 2. There is great genetic diversity between strains, even within the same type, and it has been suggested to further divide them into subgroups. For types A and B four subtypes each were found and for type E five subtypes (Hill et al., 2007).
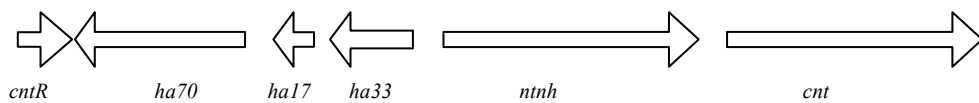
There is some confusion in literature about the names of the genes, since they have different designations in different articles. The neurotoxin gene itself was originally called *bont*, for botulinum neurotoxin, followed by type (i.e. *bontA*, *bontB*, etc). The designation *cnt*, for clostridial neurotoxin, followed by type (i.e. *cntA*, *cntB*, etc.) was suggested by the ASM Publications Board Nomenclature Committee (Lövenklev et al., 2004) (Paper II). Recently Sebaihia et al. (Sebaihia et al., 2007) suggested that all clostridial neurotoxins (including tetanus toxin) should be called *cntA*, presumably followed by toxin type. They also suggested that all non-toxin non-hemagglutinin genes, earlier known as *ntnh*, be called *cntB*. The hemagglutinin genes are known as *ha33*, *ha34* or *cntC*, *ha17* or *cntD*, and *ha70* or *cntE*. The

positive regulator is known as *p21*, *botR* or *cntR*. In type A2, E, and F, which lack hemagglutinin genes, three other open reading frames are found; *orfX1*, *orfX2*, and *p47* (East et al., 1996; Kubota et al., 1998). In this work the designations suggested by ASM will be used, except in Paper IV where the designations advocated by Sebaihia et al. are used.

Types A1, B

ha70   ha17   ha35   cntR   ntnh   cnt

Types C, D

cntR   ha70   ha17 ha33   ntnh   cnt

Type E

orfX2   orfX1   p47   ntnh   cnt

Types A2, F

orfX2   orfX1 cntR p47   ntnh   cnt

Type G

ha70   ha17 cntR   ntnh   cnt
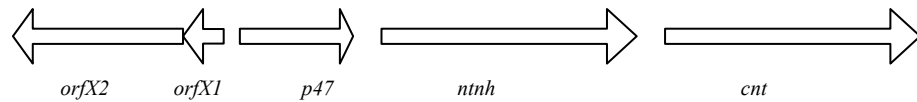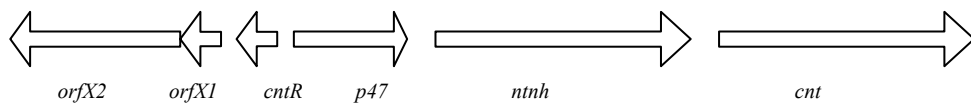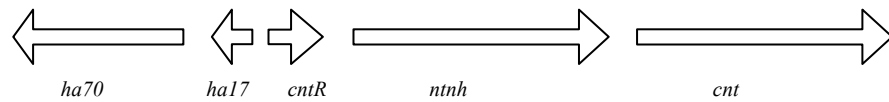
Figure 2 Organisation of the genes in the neurotoxin gene cluster. Adapted from Lövenklev (Lövenklev, 2003).

Diagnostics and detection methods

Preliminary diagnosis of botulism is based on clinical symptoms and patient history. It is probably an underdiagnosed disease, and may be misdiagnosed as myasthenia gravis, Guillain-Barré syndrome, stroke, tick paralysis, chemical intoxication, chemical or bacterial food poisoning, mushroom poisoning, medication reactions, poliomyelitis, diphtheria, or psychiatric illness. When infants are involved, meningitis, electrolyte-mineral imbalance, metabolic encephalopathy, congenital myopathy, Reye syndrome, Werdnig-Hoffman disease, and Leigh disease may also be suspected (CDC, 1998). Botulism cannot be diagnosed using routine laboratory tests, however examination of the cerebrospinal fluid (CFS) and electromyography (EMG) can help rule out other diseases. To confirm the diagnosis, identification of the toxin in samples like serum, vomitus, gastric aspirate, stool, or culture thereof is necessary.

The mouse bioassay is currently the only internationally accepted method of confirming BoNTs. Suspect material is intraperitoneally injected into mice which are monitored for botulism symptoms. The toxin type is determined using mice protected with monovalent, serotype-specific antibodies. If possible the organism is isolated. However, for food botulism laboratory tests have been reported to be positive in only 65% of the cases (CDC, 1998) and for wound botulism in the corresponding figure is only around 50% (Sieradzan, 2005). One reason for this may be that several of the commercially available test systems for identification of anaerobic bacteria have a problem with correctly identifying *C. botulinum* (Lindström et al., 1999). While the mouse bioassay is very sensitive, it has several drawbacks. It is time and labour expensive, requires a special animal facility and trained personnel, and can take up to four days. This, as well as the ethical issues of using animal testing, make the method unsuitable for large-scale testing and screening. This has led to the development of several new ways of detecting the organism and its toxin. Most toxin detection methods are based on immunological reactions, such as enzyme-linked immunosorbent assay (ELISA) (Ferreira et al., 2003), enzyme-linked coagulation assay (ELCA) (Doellgast et al., 1993), lateral flow tests (Sharma et al., 2005; Gessler et al., 2007), immuno-PCR (Wu et al., 2001), electrochemiluminescence (ECL) (Rivera et al., 2006), time-resolved fluorescence assay (Peruski et al., 2002), and biosensors (Ogert et al., 1992). Some detect the activity of the toxin, like the endopeptidase activity assay (Hallis et al., 1996; Wictome et al., 1999), endopep-MS (Kalb et al., 2006), and the neuron-based cellular assay (Dong et al., 2004; Sheridan et al., 2005). A review of the advantages and disadvantages of each was recently published by Cai et

al. (Cai et al., 2007). The main problem in testing for the toxin is that only unbound toxin can be detected. When testing for the organism, culture-based or nucleic acid-based methods, or combinations thereof are used. The nucleic acid-based methods include PCR, both conventional (Szabo et al., 1993; Franciosa et al., 1994; Fach et al., 1995; Hielm et al., 1996; Takeshi et al., 1996; Aranda et al., 1997; Alsallami and Kotlowski, 2001; Dahlenborg et al., 2001; Lindström et al., 2001; Chaffer et al., 2006) and real-time (Kimura et al., 2001; Akbulut et al., 2004; Yoon et al., 2005; Christensen et al., 2006; Fenicia et al., 2007), and DNA arrays (Song et al., 2006). Lindström et al. (2006a) have recently reviewed both culture methods, immunological methods, typing methods, and PCR protocols and compared their sensitivities, reproducibility, and ease of use. They found that a combination of methods is often needed, as each method has different strengths and weaknesses.

Diagnostic PCR

In the 20 years since the polymerase chain reaction (PCR) was first described (Mullis and Faloona, 1987), this method of selectively amplifying a DNA segment has become an increasingly important tool in microbial diagnostics. Its main advantages are that it is very rapid, specific, and can detect organisms that are hard or impossible to detect by culture (Sachse, 2003). When it comes to viruses, an improved sensitivity is observed in almost all instances when compared to culture (Cockerill, 2003; Niesters, 2004). In addition to being very specific, PCR can be used to selectively determine things like subtype, genotype, variant, mutant, and genotypic resistance patterns (Niesters, 2004). Today, diagnostic PCR is used in many fields, such as detection of genetically modified organisms (GMO), forensics, food microbiology, epidemiology, and detection of genetic diseases.

Real-time PCR

While conventional PCR requires post-amplification detection, like agarose or polyacrylamide gel electrophoresis, Southern blotting, sequencing, or ELISA (Neumaier et al., 1998), real-time PCR continually measures the accumulation of amplicon using fluorescence. This is done using either a dye that binds to all double-stranded DNA, like ethidium bromide (Higuchi et al., 1992) or SYBR Green I (Wittwer et al., 1997), or by labelling of primers (Chehab and Kan, 1989) or probes with fluorogenic compounds. There are several different types of probes, most of which use fluorescence resonance energy transfer (FRET). FRET is a spectroscopic process where energy is passed non-radiatively between molecules through dipole-dipole resonance transfer (Clegg, 1992). The fluorophore donor molecule absorbs a photon and transfers the energy to an acceptor molecule. The acceptor may be a quencher, which does not fluoresce, as is the case with hydrolysis probes (Holland et al., 1991) and molecular beacons (Tyagi and Kramer, 1996), or another fluorophore which emits light at a different wavelength, like in hybridisation probes (Wittwer et al., 1997). The distance between the donor and the acceptor molecule can be of macromolecular dimensions (10-100 Å), but the efficiency of the transfer is affected by the distance (Clegg, 1992).

Besides not needing post-amplification detection and thereby reducing the risk for carry-over or cross-contamination, real-time PCR has the advantage of being quantitative. This is because what is measured is not how much product you have at the end, when the reaction might have reached a plateau phase, but at what cycle (Ct or Cp depending on what type of instrument is used) the product reaches a detectable amount during the exponential increase of PCR product. This means it is measured in the early logarithmic stage of the amplification (Higuchi et al., 1993). There are many cases where this quantification is very useful. In virology, quantitative PCR can help determine the viral load and if intervention with pre-emptive antiviral strategies is required (Niesters, 2004), or to monitor the progression or eradication of infection (Monpoeho et al., 2002). It can be used to quantify the bacterial or viral load in food samples (Kimura et al., 2001; Wolffs et al., 2004b; Shan et al., 2005; Wolffs et al., 2005; Yoon et al., 2005) or to follow the expression of different genes (Mathews et al., 1999; Bustin, 2000; Jung et al., 2000; Schmittgen and Zakrajsek, 2000; Giulietti et al., 2001; Goerke et al., 2001; Rajeevan et al., 2001; Walker, 2001; Eleaume and Jabbouri, 2004; Lövenklev et al., 2004; Sharkey et al., 2004; Couesnon et al., 2006; Kouguchi et al., 2006; Shin et al., 2006).

When designing your assay there are many things to think about. The design of primers and probes is very important, as different primers can have 1000-fold different sensitivity for the same gene (Sachse, 2003) and they are the most important factor for deciding the selectivity of the assay. If there are primer-dimers generated this will decrease the sensitivity of the assay since it will be a competitive reaction and if SYBR green is used it will also make it difficult to separate the reactions, even though melting curve analysis can help. The PCR platform used can, as well as the type of probe and dye, influence the detection (Reynisson et al., 2006). The choice of probe type partially depends on the type of real-time PCR instrument used as some probe chemistries are more suited to particular instruments. This is especially true if multiplexing is desired, since the wavelengths emitted and detected determine how many and which dyes can be used. When multiplexing it is also important to make sure the colours can be clearly separated, not spilling over into each other's channels.

Once primers and probes are chosen the rest of the reaction can be optimised. The usual parameters that need attention are primer-, probe-, and magnesium concentrations and annealing temperature. While the amounts of dNTPs and DNA polymerase are usually standard, the type of DNA polymerase can play an important part in determining not just the amplification efficiency and detection window (Wolffs et al., 2004a), but also the robustness of the assay, since inhibition affects different DNA polymerases in different ways (Abu Al-Soud and Rådström, 1998; Löfström et al., 2004). What is optimal for one type of sample may not always be the best for another type. In some cases adding a facilitator may help overcome inhibitions (Abu Al-Soud and Rådström, 2000).

In this work, two different probe chemistries have been used. In Papers II and IV TaqMan probes (Livak et al., 1995) were used, while in Papers I and III hybridisation probes (Wittwer et al., 1997) were used. The principles of the two systems are described in Figure 3.

A *Taq*Man probe

R=Reporter
Q=Quencher
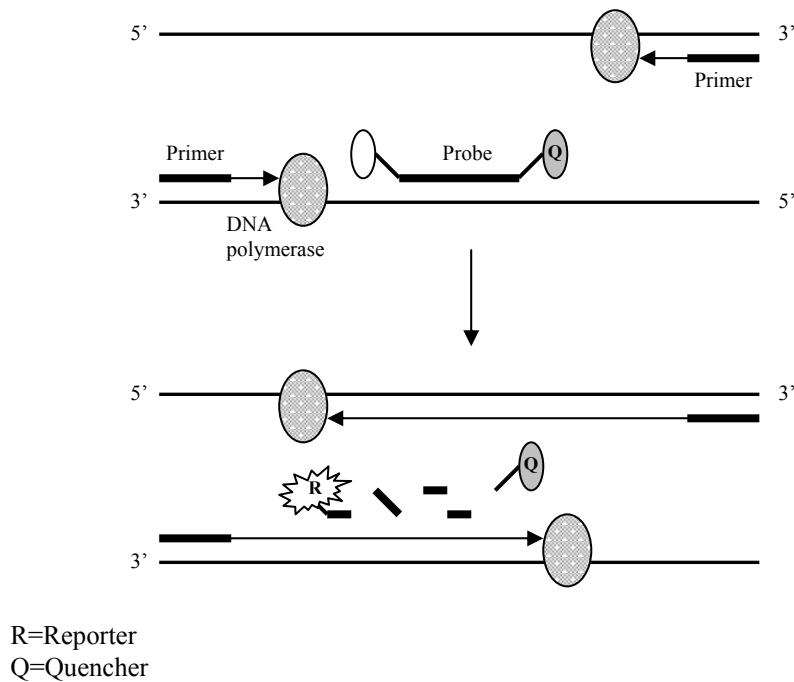
B Hybridization probe
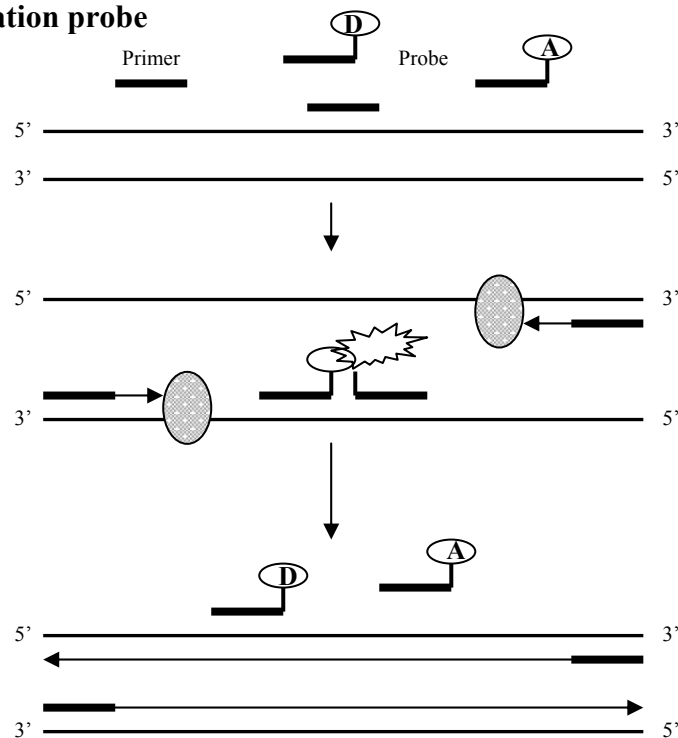
Figure 3 Principles of TaqMan probes (A) and hybridisation probes (B).

A. After hybridising to the template during the annealing step, the probe is degraded by the exonuclease activity during the extension step. When the reporter dye (R) is separated from the quencher (Q), a fluorescent signal can be measured.

B. When the two hybridisation probes hybridise close to each other, the energy from the donor probe (D) is transferred to the acceptor probe (A) through FRET, and an increase in the fluorescent signal can be measured. Used with permission of Maria Lövenklev (Lövenklev, 2003).

Internal amplification control

Many clinical and food samples contain substances which can inhibit PCR. They may do this by degrading the DNA polymerase, by denaturing it, or simply reducing the activity e.g. by binding the magnesium ions needed by the enzyme. Other inhibitors may degrade or capture the nucleic acids or, when using real-time PCR, interfere with the fluorescence. The pre-PCR processing approach used should try to eliminate these inhibitors (Rådström et al., 2004) and the use of an alternative thermostable DNA polymerase (Abu Al-Soud et al., 1998; Wolffs et al., 2004a) and amplification facilitators (Abu Al-Soud et al., 2000) can help further. The most used methods of avoiding inhibition are physical methods, like centrifugation and filtration, DNA extraction methods, and adsorption (Lantz et al., 2000). But even using all these methods there is still a risk that there will be inhibitors present in the sample that may cause a false-negative result. In order to avoid this, an internal amplification control (IAC), which is present in every tube, needs to be added to the assay. When an international standard for PCR-based detection of pathogenic microorganisms in food is being created, inclusion of an IAC has become mandatory (EN ISO 22174).
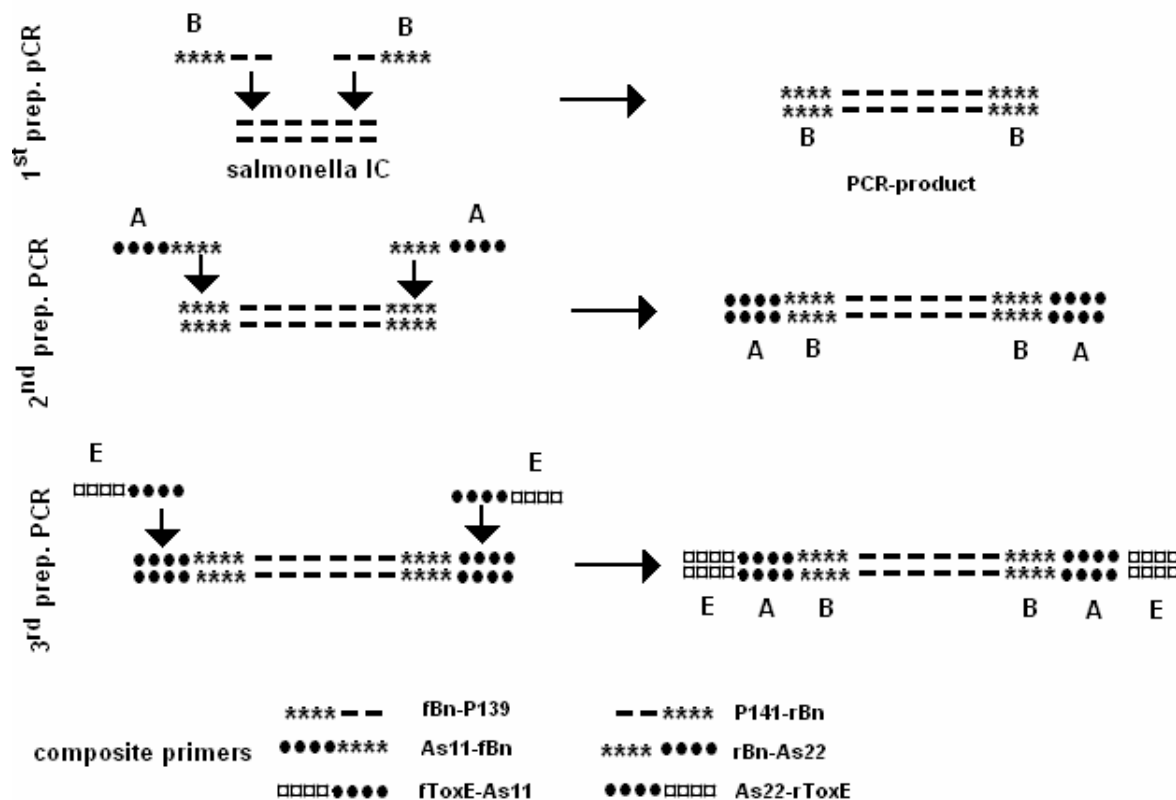

Figure 4 A schematic picture of the generation of the multiple internal amplification control.

The two main types of IACs are competitive, which uses the same primers as the target, and non-competitive, which uses different primers (Hoorfar et al., 2004). We used the method described by Stöcher et al. (2002) to generate a multiple competitive IAC for the diagnostic assays used in this thesis (Paper I). Using an existing internal control for *Salmonella* spp (IC T69), which contained a binding site for a pair of hybridisation probes (Perelle et al., 2004), as starting material, additional primer-specific sequences were added in conventional preparative PCR with composite primers (Figure 4). This was done in such a way that the amplicon from the IAC would always be larger than the amplicon from the target, since the amplification of the smaller target is favoured by the reaction kinetics (Toouli et al., 2000; Sachse, 2003; Hoorfar et al., 2004), and to be able to visually discriminate the products on a gel. Since the internal controls are used as competitors with the same primers as the clinical targets the amount of internal control present in a sample needs to be carefully titrated in order to avoid reduced amplification efficiency (Hoorfar et al., 2004). The optimal amount of IAC in the competitive PCRs was determined by comparing target detection with and without the presence of different amounts of IAC (Figure 5). While it is important to keep the amount as low as possible in order to minimise the influence on the target detection, it must be high enough to give a reproducible detection of the IAC (Rodriguez-Lazaro et al., 2004) in the samples negative for bacterial DNA as well as in the most diluted ones. We found that the amount of IAC added in our assay did not significantly change the detection limit.
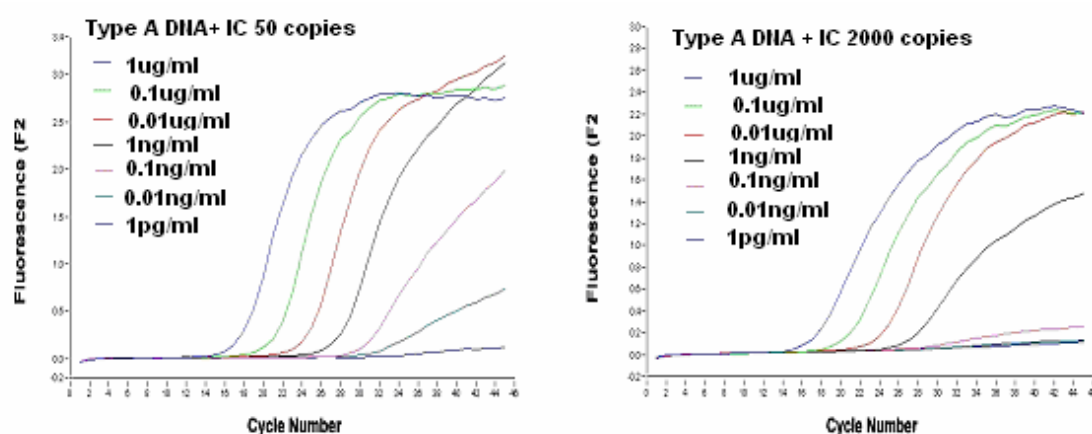

Figure 5 Effect of amount of internal control on detection of *C. botulinum* type A. When too much internal control is added the lowest concentration of target may not be detected.

Detection limits

While it is true that PCR in theory can detect as little as one DNA molecule, in practice there are several difficulties. The first one arises from the difficulty of getting that one molecule, in a sample of millilitres, or sometimes even more, into the few microlitres that are added to the reaction. Sheer probability tells us that even if there is a theoretical concentration of one molecule per volume added, not every volume taken will contain a molecule. Therefore, instead of talking about a limit of detection it is more reasonable to talk about a detection probability at a certain concentration. Thus, while the lowest amounts of DNA that could be detected for *C. botulinum* types A and E were equivalent to 0.5 and 1 genome copy respectively, the lowest concentrations that could be reliably detected corresponded to 5 and 25 copies respectively (Paper I). The detection probabilities at different DNA concentrations can be seen in Figure 2 of Paper I.

Selectivity

The selectivity of the diagnostic PCR assays were evaluated using 40 strains of *C. botulinum*, 16 strains of other *Clostridium* spp., and 26 non-*Clostridium* strains (Paper I). The selectivity is a measure of the inclusivity, i.e. the ability to detect different strains of the target pathogen, as well as the exclusivity, i.e. not detecting anything else (Malorny et al., 2003). The selectivity was found to be good, with amplification of all the correct strains, no amplification of other strains, and no cross-reaction between strains.

The problem when designing the assay is that the number of known sequences is limited, though increasing rapidly, and if the target sequence is not highly conserved there is a risk that some strains may contain variations not previously reported (Paper I). If these variations are in the area where the primers or probes are located a false-negative may be the result. This problem grows when the exclusivity is increased. While probes increase the specificity of the assay by increasing the number of places where DNA must match, this also means that there are more places where a variation can result in non-detection. This means that it is important to keep evaluating the assay as more and more strains are available for testing and more sequences are reported to sequence databases.

Types of samples

In the cell genetic information is stored as DNA. When protein production is needed the DNA is transcribed into mRNA which is then translated into polypeptides, which become the protein, after suitable post-translational modification. This gives us three different possibilities of monitoring the bacteria (Figure 6). By determining the presence of a specific gene, e.g. the toxin gene, we determine if toxigenic bacteria are present in the sample (Paper I). By measuring mRNA levels we can decide if the genes are active and determine the level of activity (Papers II, III, and IV). Finally, by measuring the concentration or activity of the toxin we can determine if the protein is being produced and released (Papers I, II, III, and IV). This can be done using *in vitro* methods like ELISA (Ferreira et al., 2003) or *in vivo* methods like the mouse bioassay (Holdeman et al., 1977).
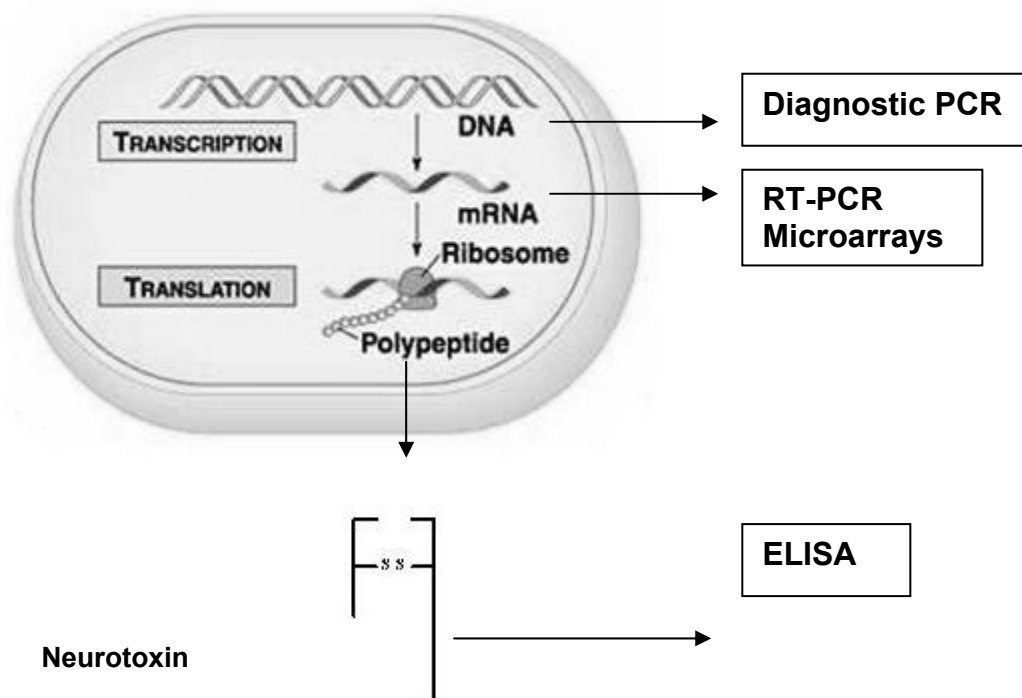

Figure 6 Different points at which samples can be taken.

Neurotoxin expression during growth

Reverse transcription PCR (RT-PCR)

For a long time molecular biologists were convinced that the flow of genetic information is unidirectional, from DNA to RNA to protein (Culliton, 1971). However, in 1964 it was found that cells infected with Rous sarcoma virus, an RNA virus, contained new DNA homologous to the viral RNA (Temin, 1964). Six years later, two independent groups found evidence of the existence of an enzyme which can synthesise DNA from an RNA template (Baltimore, 1970; Temin and Mizutani, 1970). In 1971 the RNA-dependent DNA polymerase, or reverse transcriptase as it is now known, from the avian myeloblastosis virus was purified and characterised (Kacian et al., 1971) and it was shown that it would accept genetically unrelated RNA molecules as a template (Spiegelman et al., 1971). This meant that the same enzyme could be used to reverse transcribe many different types of RNA, which could in turn be used as a template for PCR (Rappolee et al., 1989). It is possible to do this in a one-step protocol, where the reverse transcription and PCR amplification takes place in the same tube. This has, however, been seen to be less sensitive than using a two-step procedure (Bustin, 2002). An oligonucleotide primer is required to initiate the synthesis of the complementary DNA (cDNA). The first ones described were oligo(dT) primers (Rappolee et al., 1988), which will reverse transcribe all messenger RNA (mRNA) from eukaryotes. This approach is not possible when working with prokaryotes as they do not produce mRNA with a polyA-tail. The other alternatives are random hexadeoxynucleotide primers (Noonan and Roninson, 1988), which will also reverse transcribe all RNA, and specific primers (Chelly et al., 1988), which will only reverse transcribe RNA from the gene of interest. The advantages and disadvantages of each were recently reviewed by Bustin et al. (Bustin et al., 2005). In 1989 it was shown that reverse transcription PCR (RT-PCR) could be used to quantify specific mRNA sequences in a reproducible way (Becker-Andre and Hahlbrock, 1989; Delidow et al., 1989).

In the present work quantitative RT-PCR (qRT-PCR) methods were developed to monitor the *cntB* expression in *C. botulinum* type B (Paper II), *cntE* expression in *C. botulinum* type E (Paper III) and *cntA* and *cntR* expression in *C. botulinum* type A (Paper IV). We use a two-step procedure where the mRNA is first reverse transcribed using Superscript II and specific primers, then quantified with real-time PCR using *Tth* polymerase and probes. This system

was developed after comparing several different methods, both one-step and two-step, and enzymes.

Real-time PCR quantification

As mentioned earlier, real-time PCR is quantitative. There are several different ways this can be used. Absolute quantification can be performed by comparing Ct values to a standard curve constructed with known concentrations of target. This is more useful when quantifying DNA than mRNA, since a pre-constructed standard does not take differences in RT efficiency into account. While it would be possible to include RNA standards in the RT step, insuring the same transcription efficiency, there is a problem with the stability of the standards, as RNA tends to degrade faster than DNA. This means that the method is generally only used when absolute quantification is essential, like in quantifying viral loads. The principle behind a standard curve can be seen in Figure 7.
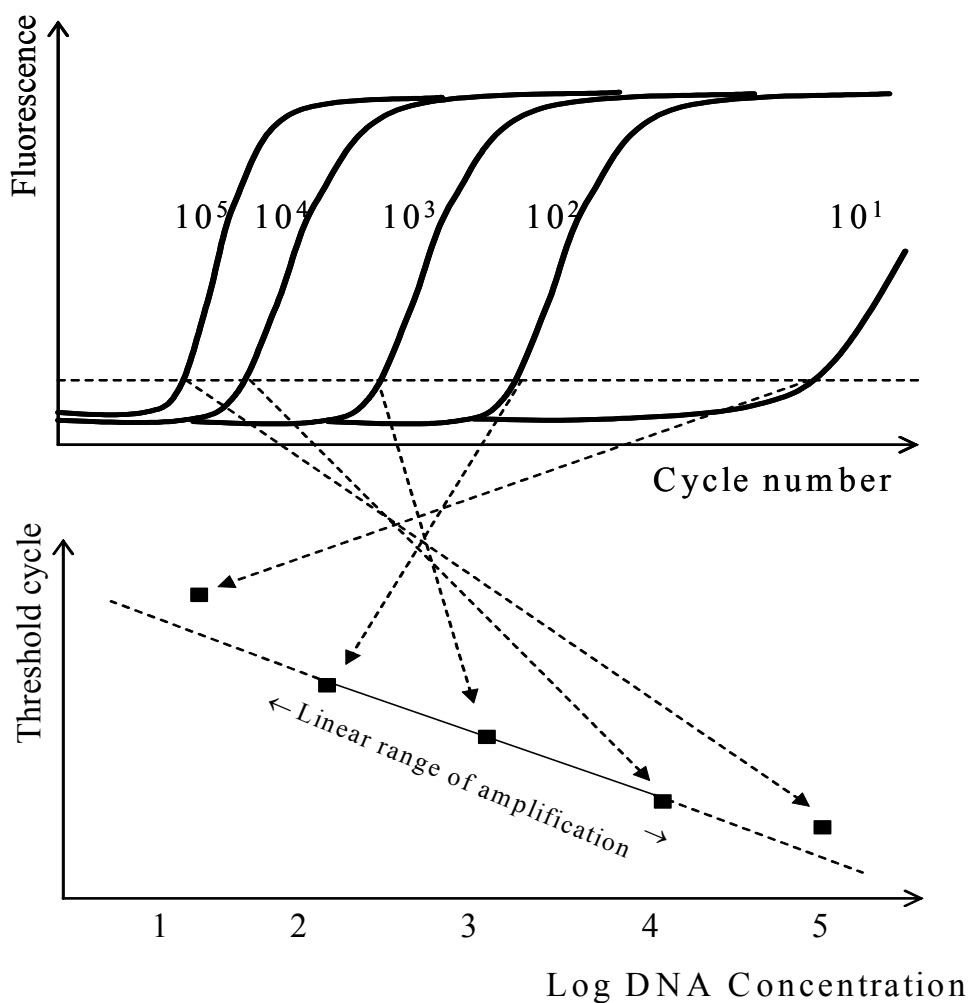

Figure 7 Schematic picture of standard curve. While product can be detected in the entire detection window, quantification assuming linear relationship is only valid in the linear range of amplification. Used with permission of Petra Wolffs (Wolffs, 2004).

Relative quantification can be performed by correcting for differences in RNA input or reverse transcription efficiencies by normalising them to a housekeeping gene. This should be a gene that is expressed at a constant level in all the relevant times and conditions tested (Giulietti et al., 2001). Another method for relative quantification is the comparative Ct method. In the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001) the amount of target relative to a calibrator and normalised to a housekeeping (reference) gene is given by

$$\text{amount of target} = 2^{-\Delta\Delta Ct}$$

$$\Delta\Delta Ct = \Delta Ct(\text{sample}) - \Delta Ct(\text{calibrator})$$

$$\Delta Ct = Ct(\text{target}) - Ct(\text{reference})$$

The biggest problem with this method is that it requires close to identical amplification efficiencies of target and housekeeping gene. While efficiencies close to 100% should always be sought, it is not always possible to achieve this. Pfaffl (Pfaffl, 2001) solved this problem by including the amplification efficiency in his formula for calculating relative expression (RE)

$$RE = ((1+E_{target})^{\Delta Ct_{target}^{(\text{calibrator- sample})}}) \,/\, ((1+E_{reference})^{\Delta Ct_{reference}^{(\text{calibrator- sample})}})$$

The amplification efficiency (E) is

$$E = (10^{(-1/s)}) - 1$$

where s is the slope of the log-linear range of amplification (Figure 7) (Klein et al., 1999).

Microarrays

A new method of gene expression profiling is beginning to play a bigger part as more and more genomes are being sequenced. DNA microarrays, membranes or glass slides with a mosaic of the entire genome, either as oligonucleotides (oligonucleotide microarrays) or as PCR products representing the individual genes (cDNA microarrays), give the possibility to assess transcription on a genomic scale. The two technological advances mainly responsible for the development of microarrays are application of robotic technology to routine molecular biology and dual fluorescent labelling (Lucchini et al., 2001). In addition to being used to examine expression microarrays can be used for genome typing and to study microbial evolution. A microarray based on the genome of the *C. botulinum* type A strain ATCC 3502 has recently been used for comparative genomic indexing (Sebaihia et al., 2007). This is the same type of microarray used in Paper IV. Figure 8 illustrates the placement of the array probes in the toxin gene cluster.

Briefly, reference and sample DNA or cDNA is labelled, one with Cy3, the other with Cy5. They are then simultaneously hybridised to the microarray slide, which thus is unaffected by variability in hybridisation or differences between individual microarrays (Lucchini et al., 2001). After hybridisation the ratio between the amount of reference and sample that has hybridised can be read with a microarray scanner and the digital image can be processed. Grids specifying the location of the target genes can be overlaid on the images (Duggan et al., 1999). Either DNA or cDNA can be used as a reference. In this work genomic DNA was used as a reference (Paper IV). Labelling the mRNA can either be done by first reverse transcribing it, then labelling it the same way the reference DNA is labelled, or by directly labelling it during the reverse transcription. We chose the direct labelling approach, in order to minimise the effect of any remaining DNA in the RNA preparation (Paper IV). An overview of the setup can be seen in Figure 9.

By studying the transcriptome, i.e. changes of gene expression and transcription on a global scale, a vivid picture of the cell's adaptation to a changing environment can be seen. Furthermore, the behaviour of large groups of functionally related genes can be studied and common regulatory functions found for sets of genes which show similar expression profiles (DeRisi et al., 1997).

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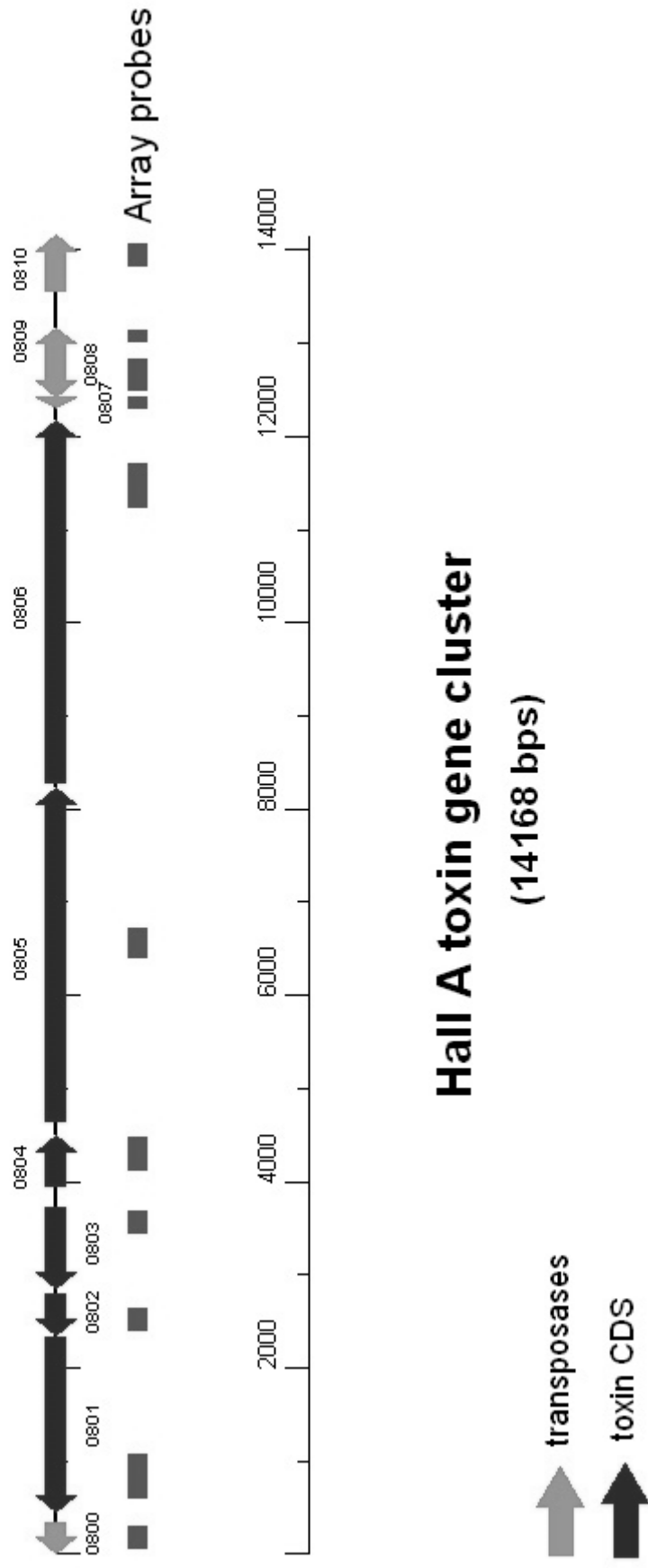
Figure 8 Organisation of genes and microarray probes of the neurotoxin gene cluster in ATCC 3502. The numbering of the coding sequences is according to Sebaihia et al. (Sebaihia et al., 2007). CBO0800 - putative transposase (partial), CBO0801- hemagglutinin component of the neurotoxin complex, CBO0802 - hemagglutinin component of the neurotoxin complex, CBO0803 - hemagglutinin component of the neurotoxin complex, CBO0804 - component of the neurotoxin complex (cntR), CBO0805 - type A progenitor toxin nontoxic-nonhemagglutinin, CBO0806 - botulinum neurotoxin type A precursor, CBO0807 - transposase (pseudogene), CBO0808 - transposase/integrase, CBO0809 - putative transposase (pseudogene), CBO0810 - putative transposase for insertion sequence element IS904. Used with permission of Andrew Carter (IFR, Norwich, UK).

28

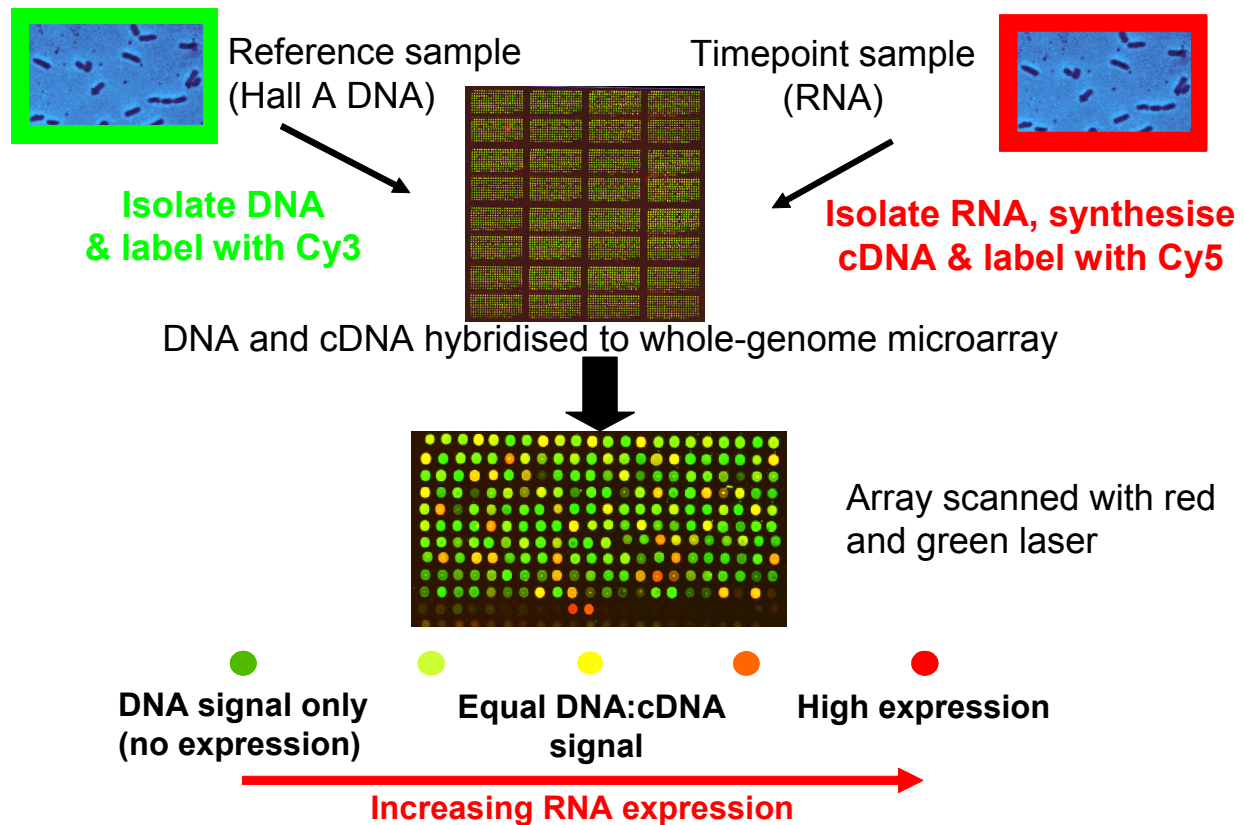**Figure 9** Setup for Hall A microarray analysis. The reference DNA is labelled with Cy3 and the sample cDNA with Cy5. Each array contains approximately 3500 gene probes spotted in duplicate, arranged in 16 blocks. Each slide bears two arrays. Used with permission of Mike Peck and Andrew Carter (IFR, Norwich, UK).

Neurotoxin gene expression profiles

Little is known about the direct regulation of the neurotoxin gene (*cnt*), as most studies have focused on germination, growth and time to toxicity, using the mouse bioassay. However, knowing when a gene is expressed is often a strong indicator of its biological role. While regulation of mRNA is not the only way abundance of protein is regulated, most differences in cell types or states are correlated to changes in mRNA levels of several genes. A positive regulator, *cntR*, has been found in all *C. botulinum* types except type E. In this work we have followed the expression profiles of *C. botulinum* types A, B, and E during growth in different conditions (Papers II, III, and IV). In the case of *C. botulinum* type A we also followed the expression profile of *cntR* and the genome-wide expression using microarrays (Paper IV).
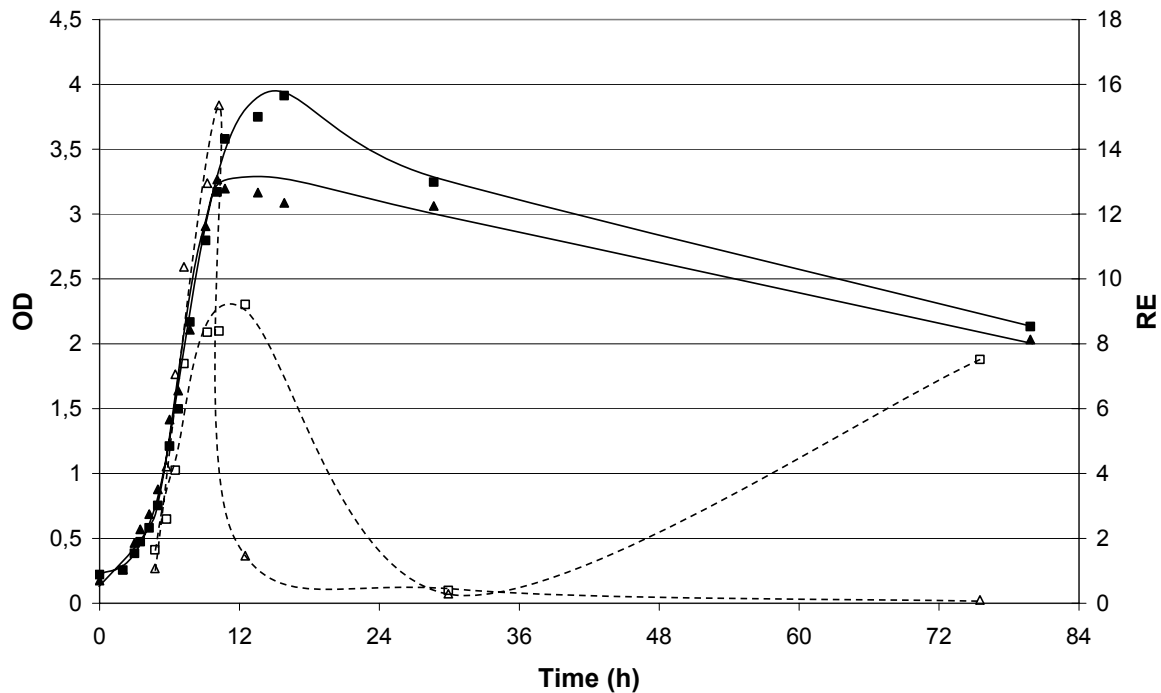
Figure 10 Growth and expression in *C. botulinum*. Squares – proteolytic type A (Hall A), triangles – non-proteolytic type E (ATCC 9564), whole lines – OD_{620}, dashed lines – relative neurotoxin expression.

When looking at the profiles of neurotoxin expression during the different growth stages it is easy to see some similarities between the different neurotoxin types of *C. botulinum*, as well as certain differences. They all seem to have a maximum relative *cnt* mRNA level during late exponential or early stationary phase and then to decline, but while type E goes down to a very low level and then stays there (Figure 10), some of the other types show secondary mRNA peaks in later phases. In some cases these peaks seem as high as or possibly even higher than the first peak. This behaviour seems more marked in proteolytic than in non-proteolytic strains. In the proteolytic type B strain ATCC 17841, Lövenklev et al. (Lövenklev et al., 2004) seem to see two later peaks, one, higher than the first one, at 11 h and one, approximately the same as the first one, at 30 h, while the other strains only showed the one at 30 h. The levels of expression differ between the different strains, with proteolytic strains generally showing higher levels. The highest expression levels and the highest concentrations of toxin were found in the proteolytic strain ATCC 7949. In proteolytic *C. botulinum* type A strain Hall A there was clearly a second peak in late stationary or death phase. It was in the same range as the first peak, and may have continued to rise.

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Table 2 Genes with expression similar (P≥0.99) to neurotoxin gene cluster.

Locus	P-value	Protein
CBO0026	0.996	Membrane protein
CBO0433	0.990	Hydrogenase nickel incorporation protein
CBO0575	0.990	Two-component response regulator
CBO0644	0.990	Conserved hypothetical protein
CBO0909	0.995	GnaT-family acetyltransferase
CBO1134	0.990	Pyrazinamidase/nicotinamidase
CBO1238	0.996	Conserved hypothetical protein
CBO1254	0.997	Thioredoxin reductase
CBO1288	0.995	Guanine deaminase
CBO1402	0.997	ABC transport system, ATP-binding protein
CBO1815	0.991	Conserved hypothetical protein
CBO1848	0.992	Electron-transferring subunit of iron-only hydrogenase
CBO1849	0.993	Electron-transferring subunit of iron-only hydrogenase
CBO2021	0.990	Chloramphenicol acetyltransferase
CBO2079	0.997	Transmembrane protein
CBO2264	0.991	Transporter protein
CBO2316	0.994	Membrane protein
CBO2473	0.992	Integral membrane protein
CBO2948	0.999	Bifunctional membrane-associated kinase and phosphatase
CBO3515	0.995	Translation elongation factor G, Ef-G

The peak in late exponential phase has been seen in several strains of *C. botulinum* type A (Bradshaw et al., 2004; Couesnon et al., 2006; Shin et al., 2006), with strain KNIH-1 as the only exception (Shin et al., 2006), but the second peak is only mentioned as a "slight transient increase" by Couesnon et al. (2006) and not at all by the others. It may be that those strains did not have such a marked second peak, or that they did not follow the expression for a long enough period to detect it. It may also be an effect of the different medias used, as this is known to influence expression (Bradshaw et al., 2004). In Hall A the positive regulator gene *cntR* was also studied with real-time PCR. The expression profile was, as expected, very similar to the one of the gene complex it regulates, even if it was expressed at much lower levels.

When studying the genome-wide gene expression in Hall A with microarrays we found that the entire neurotoxin gene cluster is expressed as a whole, with similar expression levels, except for the positive regulator gene, *cntR*, which is expressed at a much lower level, CBO0800 (transposase), which is expressed at a higher level, and CBO0803 (HA34), which does not seem to follow any pattern at all (Paper IV, Figure 4). The latter may be because of problems with the probe, since Couesnon et al. (Couesnon et al., 2006) claim it follows the same pattern as the rest of the cluster, but at higher levels. There are several other genes that follow the same expression pattern as the toxin gene cluster (Table 2). Other genes are obviously different, many showing little variation in expression throughout the growth curve. Most of the other genes follow the predicted pattern, such as activation of the sporulation genes in the later parts of the growth curve (Paper IV, Figure 10).

Effect of CO$_2$

Modified atmosphere packaging

During the last decades modified atmosphere packaging (MAP) of food has become increasingly common. The main reason for this has been consumer demand for fresh and chilled products, preferably without preservatives, which will still keep for a long time. From the industry's side the extended shelf life means economic gains since packaging can be centralised and new types of products, like refrigerated ready-to-eat foods, can take the place of the declining canned and frozen foods (Farber, 1991). MAP is used for many different types of food, like fruits, vegetables, eggs, meat, bakery products, and seafood, as well as for ready-to-eat foods.

The three gases most used in MAP are oxygen (O$_2$), nitrogen (N$_2$), and carbon dioxide (CO$_2$). Oxygen is used mainly in MAP of meats to maintain the bright red colour and to prevent the growth of anaerobic bacteria (Farber, 1991). While *C. botulinum* is considered an anaerobic organism, spores can germinate in $\leq$1% O$_2$ and vegetative cells can grow in 15% O$_2$ (Whiting and Naftulin, 1992), showing that inclusion of oxygen is not enough in itself.

Nitrogen is used as a filler gas and to displace oxygen, in order to delay oxidative rancidity and growth of aerobic spoilage bacteria. It is not antimicrobial in itself and it has been shown that it will allow *C. botulinum* to grow, while it retards spoilage (Kautter et al., 1981).

Carbon dioxide is the gas responsible for most of the retardation of microbial growth. It is most effective for foods whose spoilage flora consists of gram-negative, aerobic, psychrotrophic bacteria (Daniels et al., 1985). The mechanism for its effect is not completely understood and several theories have been suggested. While reducing the available oxygen by displacing it would interfere with aerobic organisms, CO$_2$ also affects anaerobes, and replacing it with 100% N$_2$ does not have the same effect, indicating that this is not the main reason for its effect. Other theories include an alteration of the cell membrane, leading to decreases in glucose uptake rates and amino acid absorption, inhibition of enzymes, or induction or repression of their production, penetration of the cell membrane, leading to intracellular pH changes, and direct changes to proteins, like their solubility, reactivity, stability, charge, or configuration (Daniels et al., 1985; Dixon and Kell, 1989). It is generally agreed that it is the CO$_2$ dissolved in the fat and water phase that is effective. The amount that can be dissolved in different foods depends on several different factors both intrinsic, like pH and fat content, and extrinsic, like storage temperature, gas/product volume ratio and

concentration of CO_2 in the gas phase (Devlieghere et al., 1998a). The solubility of CO_2 in the water phase, at moderate temperatures and pressures, closely follows Henry's law (Daniels et al., 1985):

$$[CO_2]_{diss} = K_H \times pCO_2$$

where K_H is Henry's law's constant and pCO_2 is the partial pressure of CO_2. Since K_H varies with temperature, more gas can be dissolved at lower temperatures. There also seems to be a synergistic effect between CO_2 and temperature, increasing the antimicrobial activity more than can be explained by just the increase in dissolved CO_2 (Devlieghere et al., 1998b).

In spite of all the advantages with MAP there is a valid concern that the modified atmosphere may inhibit spoilage organisms, while allowing or even promoting growth of pathogens. This might mean that food appears unspoiled and organoleptically acceptable, even long after the expiration date, while it is in fact toxic. This is especially a hazard in ready-to-eat foods which are not heated before consumption. The organisms that mainly cause concern in refrigerated foods are psychrotrophic foodborne pathogens such as *Listeria monocytogenes*, *Yersinia entercolitica* and *Aeromonas hydrophila,* as well as non-proteolytic *C. botulinum*, although clearly a number of other microorganisms, especially *Salmonella* spp., *E. coli* O157:H7 and *Shigella* spp., can be potential health risks. Another type of food where MAP is considered hazardous is fresh fish. This is in part because of the high prevalence in fish of spores of *C. botulinum* type E. Since these non-proteolytic strains can produce toxin at temperatures as low as 3.3˚C, and since maintenance of a storage temperature that is lower from time of packaging to consumption is not likely with current practices and abuses at the retail and consumer level, the US Food and Drug Administration (FDA) considers these products "a potentially life threatening acute health hazard because of the possibility of contamination with *C. botulinum* toxin". They have ordered a detention without physical examination of refrigerated (not frozen), vacuum or modified atmosphere packaged raw fish and fishery products since "FDA considers refrigerated fresh fish products in vacuum packaging or modified atmosphere packaging to be adulterated under section 402(a)(4) of the Food, Drug and Cosmetic Act when the *C. botulinum* toxin hazard is not controlled" (IA #16-125 - 9/25/02). In Canada, regulation number B.21.025 of the Regulations Pursuant to the Food and Drugs Act states that "No person shall sell smoked marine and fresh water animals and their products or marine and fresh water animals and their products to which liquid smoke flavours has been added that is packed in a container sealed to exclude air unless it a) has been heat processed after sealing at a temperature and for a time sufficient to destroy all spores of the species *Clostridium botulinum*; or b) contains not less than 9 percent salt, as

determined by the official method; or c) is customarily cooked before eating; or d) is frozen and the label carries the following statement on the principal display panel in the same size type as used for the common name "Keep Frozen Prior to Use"." Neither the EU in general nor Sweden has any rules limiting the use of CO_2 in food packaging. It is listed as substance generally permitted to be added to all foodstuffs in European Parliament and Council Directive No 95/2/EC on food additives other than colours and sweeteners and the Swedish law on food additives, LIVSFS 2004:30. The product needs to be marked "Packed in a protective atmosphere", but the contents of the gas do not have to be specified.

Several investigations have shown that toxin production by *C. botulinum* can precede spoilage in such diverse foods as fish (Arritt et al., 2007), crumpets (Daifas et al., 1999a; Daifas et al., 1999b), rice (Kasai et al., 2005), sandwiches (Kautter et al., 1981), pork (Lambert et al., 1991), and sliced raw potatoes treated with $NaHSO_3$ (Solomon et al., 1998) when stored under modified atmosphere.

Effects of carbon dioxide on growth, toxin gene expression, and production of neurotoxin

While most studies have focused on the time to germination, growth, or toxin production under different modified atmospheres and at different temperatures (Kautter et al., 1981; Doyle, 1983; Foegeding and Busta, 1983; Lambert et al., 1991; Solomon et al., 1998; Daifas et al., 1999a; Daifas et al., 1999b; Gibson et al., 2000; Fernandez et al., 2001; Kasai et al., 2005; Arritt et al., 2007), we have concentrated on quantifying the neurotoxin expression and the amounts of toxin produced during different growth phases.

We found that the effect of carbon dioxide varies with the toxin type and possibly group. The effect on the expression of the different toxin types can be seen in Figure 12. While the maximum OD did not seem negatively affected, in non-proteolytic type B and E, growth rates decreased with increasing concentrations of CO_2 and lag phases increased (Papers II & III) (Figure 11).
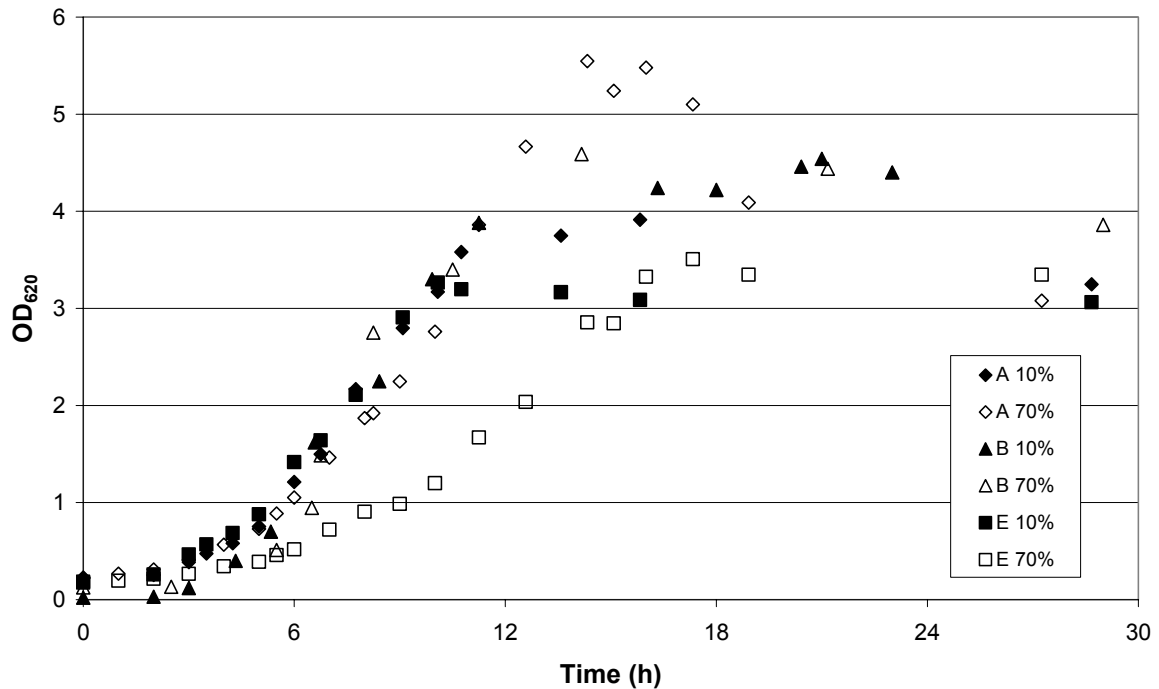
Figure 11 Effect of carbon dioxide on growth of *C. botulinum*. Diamonds – *C. botulinum* type A. Triangles – *C. botulinum* type B. Squares – *C. botulinum* type E. Filled symbols – 10% CO_2. Open symbols – 70% CO_2.

The neurotoxin gene expression, however, behaved in the opposite way, increasing with the level of CO_2, as did the amount of toxin produced. For *cntE* the expression in late exponential phase more than doubled when the concentration of CO_2 increased from 10% to 70%, while for *cntB* the effect was even more dramatic as it increased five-fold (Figure 12). The amount of toxin produced increased in a similar manner. While Sharkey et al. (Sharkey et al., 2004) claim that increased growth rate and cell mass production generally increases the levels of toxin encoding mRNA and people traditionally have believed that slowing growth will decrease toxin production, this phenomena is not completely unknown among clostridia. *Clostridium difficile* has been shown to produce more toxin when growth is slowed by stressful events (Moncrief et al., 1997). Since the expression of toxin in *C. difficile* is induced when it reaches stationary phase (Dupuy and Sonenshein, 1998), this may not be completely analogous. However, in both cases it has been suggested that one purpose of producing toxins may be to kill eukaryotic organisms, so the bacteria can utilise their nutrients and energy. Similar trends have been found in other genera as well. Expression of *emm*, the gene encoding M protein, and *scpA*, encoding the C5a endopeptidase Scp, which are major virulence factors in *Streptococcus pyogenes*, were stimulated by increased levels of CO_2 (Caparon et al., 1992; Stretton and Goodman, 1998). Carbon dioxide has also been found to stimulate toxin

production in *Staphylococcus aureus, Vibrio cholerae,* and *Bacillus anthracis* (Shimamura et al., 1985; Hoffmaster and Koehler, 1997; Stretton et al., 1998; Ross and Onderdonk, 2000; Drysdale et al., 2004; Drysdale et al., 2005).
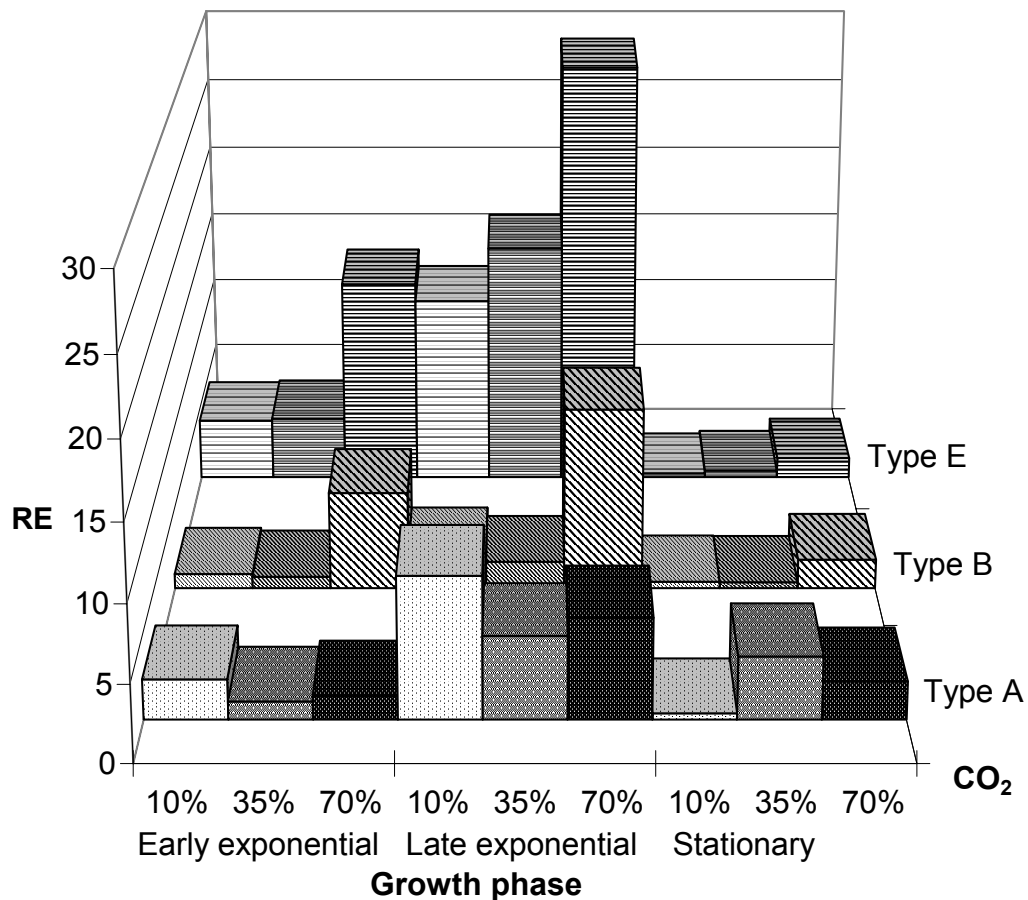

Figure 12 Effect of growth phase and carbon dioxide concentration on relative expression of neurotoxin in *C. botulinum.*

In the proteolytic type A, a completely different picture could be seen (Paper IV). Both the growth and the toxin expression seem unaffected by the amount of CO_2 in the atmosphere, even if the maximum OD seems to rise with increasing CO_2 levels. When looking at the overall expression of Hall A with microarrays, the trend is similar, but the expression of the neurotoxin cluster appears to continue rising, except for the *cntR* gene, which indicates that there may be something else affecting expression in the late stationary/death phase. While the increase in CO_2 does not seem to have a significant effect on the toxin expression, several other genes were induced at 35% and 70% CO_2. A list of these genes can be seen in Table 3. Several of these genes have been seen to be involved with stress response (Nagasawa et al., 1988; Kawasaki, 1993; Chen et al., 1998; Campobasso et al., 2000; Uo et al., 2002;

Goldsmith-Fischman et al., 2004; Glantzounis et al., 2005; Oliveira et al., 2005; Obiero et al., 2006; Schwarz and Mendel, 2006).

Table 3 Genes induced by growth at 35% and 70% CO_2.

Locus	Protein
CBO2881	Xanthine dehydrogenase, molybdenum-binding and iron-sulfur-binding subunits
CBO2882	Peptidase
CBO2883	Amidohydrolase
CBO2884	Pyridine nucleotide-disulfide oxidoreductase
CBO2885	Aspartate/ornithine carbamoyltransferase
CBO2886	D-hydantoinase
CBO2887	Xanthine permease
CBO2888	Diaminopropionate ammonia-lyase
CBO1259	Thioredoxin reductase
CBO1848	Electron-transferring subunit of iron-only hydrogenase
CBO0450	Hydroxyethylthiazole kinase
CBO2221	Initrogenase iron-molybdenum cofactor protein
CBO2222	4Fe-4S cluster containing ParA family ATPase protein
CBO2223	4Fe-4S cluster containing ParA family ATPase protein

Concluding remarks

The research presented in this thesis has been focused on developing molecular methods for diagnosis of botulism and to increase the understanding of *Clostridium botulinum* and its neurotoxin formation. This knowledge can be used to improve food safety assessment, by seeing how different factors affect food preservation.

While methods to preserve food in a way that prevents botulism have been known for a long time, new consumer demands, new health issues, and new behavioural patterns create new problems. Today's consumers want food with little or no preservatives, salt, or sugar. This means that food producers try to cut down on salt and sodium nitrite, which has long been the combination of choice for preventing growth of *C. botulinum* in foods that could not be put through a botulinum cook (a 12D reduction in the amount of spores). At the same time the consumers want food with a long shelf life, that they can keep at home for a long time, without spoilage, and preferably without refrigeration. And lastly, they want food which looks and tastes fresh, with vitamins and other nutrient intact, which means it can not have undergone a harsh heat treatment. All this, taken together, has caused the food industry to try to find new methods of preserving food. While the new methods are tested for their ability to stop the growth of spoilage organisms, it is not certain that all types of pathogens are taken into account. As *C. botulinum* spores are present in a variety of places, including soil, fish, and the intestines of pigs and cattle (Dodds, 1993b; Dahlenborg et al., 2001; Dahlenborg et al., 2003), there is a high risk of contamination if the food is not handled correctly.

The safety of some of the new methods used for preservation, like MAP and vacuum packing, are dependent on the cold chain being kept. However, it is a well known fact that it is often broken, during transport to stores, by being left on the loading bay, by incorrect temperatures in the stores, and not least by the consumers themselves. Many consumers are under the impression that since something is vacuum packed it doesn't matter if it doesn't go straight in the refrigerator. Furthermore, most household refrigerators do not keep cold enough. Sous-vide (ready to eat, vacuum packed food) meat is e.g. supposed to be stored at a temperature below 3°C, a temperature seldom held in a household refrigerator.

In this work we found storing food under a high concentration of carbon dioxide can even increase the risk of botulism, since the expression and production of neurotoxin for non-proteolytic *C. botulinum* was enhanced, in spite of the delay in growth. Since these are the types that can grow at refrigeration temperatures, this can prove to be a great hazard. In the case of the proteolytic type A, neither growth nor toxin production was influenced. It would

be interesting, in the future, to see if this is a true difference between group I and II organisms, or something which varies between strains.

Finding new, quicker ways of identifying botulism can be effective in shortening the length and cost of hospital stay and the severity of illness. In addition, administration of antitoxin is most effective if done quickly. All types of botulism are probably underdiagnosed, partially because of problems in laboratory identification. In many cases, antibiotics are given before samples are taken, which prevents normal culture methods, while DNA-based methods can still be used. Because botulism is a relatively rare disease, delayed diagnosis is not uncommon. Because all types of botulism are relatively rare diseases and delayed diagnosis is not uncommon it is important that clinicians are aware of their existence.

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