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# **Neurotoxin regulation and the temperature stress response in *Clostridium botulinum***

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ACADEMIC DISSERTATION

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## Abstract

*Clostridium botulinum* is a dangerous foodborne pathogen that forms highly resistant endospores and the extremely potent botulinum neurotoxin. Whereas endospores enable the survival and transmission of the organism in many harsh environments, the botulinum neurotoxin blocks neurotransmission and causes the severe and potentially lethal disease botulism in humans and animals. Both traits play an important role in the life of this pathogen and temporally overlap in *C. botulinum* batch cultures, suggesting common regulation. However, the co-regulation of sporulation and neurotoxin synthesis and the significance of both traits during stress conditions have not been examined in detail. This study focused on the role of the master regulator of sporulation Spo0A in neurotoxin synthesis in Group II *C. botulinum* type E strains, which lack the well-known neurotoxin gene regulator BotR. Furthermore, the role of the two traits during heat stress in Group I *C. botulinum* ATCC 3502 was investigated. Group II *C. botulinum* strains represent the main hazard in minimally-processed anaerobically-packaged foods relying on cold storage, as Group II strains are able to grow and produce toxin at temperatures as low as 3 °C. Cold tolerance mechanisms are scarcely known in psychrotrophic Group II *C. botulinum*. Studying the mechanisms required for adaption and growth at low temperatures is crucial to counter the safety hazards caused by this dangerous pathogen. The role of a two-component signal transduction system in the cold tolerance of Group II *C. botulinum* type E was investigated. A better understanding of neurotoxin gene regulation and mechanisms contributing to cold tolerance might enable the development of measures to reduce the risk of botulism outbreaks.

The sporulation transcription factor Spo0A was shown to control the initiation of sporulation and neurotoxin synthesis in *C. botulinum* type E Beluga. The non-sporulating *spo0A* mutants produced drastically less neurotoxin than the wild-type strain, and *in vitro* binding assays showed that Spo0A binds to a putative Spo0A-binding box (CTTCGAA) upstream of the neurotoxin gene operon, suggesting the direct activation of neurotoxin synthesis by Spo0A. The sequence and location of the putative Spo0A-binding box is conserved among *C. botulinum* type E strains, and analysis of *spo0A* mutations in two more type E strains (K3 and 11/1-1) affirmed the important role of Spo0A in neurotoxin type E synthesis. Spo0A is the first neurotoxin regulator reported in *C. botulinum* type E strains that lack the neurotoxin gene activating alternative sigma factor BotR. However, co-regulation of sporulation and neurotoxin synthesis is probably not limited to type E strains. Analysis of heat shocked continuously growing *C. botulinum* type A ATCC 3502 cultures revealed simultaneous downregulation of both traits in response to heat stress, which was affirmed by decreased toxin synthesis and abolished sporulation in batch cultures incubated at 45 °C compared to cultures incubated at 39 °C. This suggests that both traits might be co-regulated in *C. botulinum* type A, possibly also via Spo0A, which was significantly downregulated after heat shock, whereas the expression of genes encoding the known neurotoxin gene transcription activators BotR and CodY was unaffected or even upregulated during the heat shock response. While heat stress had a negative effect on sporulation and neurotoxin synthesis, the expression of genes related to motility was

induced after heat shock. This suggests that motility is the preferred choice when facing elevated temperatures, probably to search for environments with less harmful temperatures.

In order to grow and produce neurotoxin at cold temperatures, bacteria have to sense low temperatures and adjust their metabolism and structure for efficient growth in cold environments. We identified the first two-component signal transduction system (TCS) induced during the cold-shock response and needed for efficient growth at low temperatures in psychrotrophic *C. botulinum* type E. Expression of the TCS genes *clo3403* (encoding a histidine kinase for sensing) and *clo3404* (encoding a DNA regulator for responding) was increased after cold shock and prolonged compared to the expression pattern observed at the optimal growth temperature, suggesting that the TCS CLO3403/CLO3404 is needed for cold adaptation. Furthermore, inactivation of the TCS genes *clo3403* and *clo3404* resulted in impaired growth with significantly reduced maximum growth rates at low temperatures but not at the optimum temperature compared to wild-type growth. The important role of the TCS CLO3403/CLO3404 for cold tolerance in *C. botulinum* type E was confirmed by successful complementation of the mutations.

In summary, this study demonstrated that sporulation and neurotoxin synthesis are co-regulated via the master regulator of sporulation Spo0A in *C. botulinum* type E and that heat stress has a negative effect on both traits in *C. botulinum* ATCC 3502, which also suggests common regulation in type A strains. Sporulation-dependent neurotoxin synthesis might play a central role in the life of this dangerous pathogen and represents a key intervention point for control. Finally, we identified a TCS (CLO3403/CLO3404) important for cold adaptation in psychrotrophic *C. botulinum* type E, which represents a major hazard in anaerobically-packaged chilled foods.

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## List of original publications

This thesis is based on the following publications:

- I Mascher, G., Mertaaja, A., Korkeala, H., Lindström, M. 2017. Neurotoxin synthesis is positively regulated by the sporulation transcription factor Spo0A in *Clostridium botulinum* type E. *Environ Microbiol.* 19(10):4287-4300.
- II Selby, K., Mascher, G., Somervuo, P., Lindström, M., Korkeala, H. 2017. Heat shock and prolonged heat stress attenuate neurotoxin and sporulation gene expression in group I *Clostridium botulinum* strain ATCC 3502. *PLoS One.* 12(5):e0176944.
- III Mascher, G., Derman, Y., Kirk, D. G., Palonen, E., Lindström, M., Korkeala, H. 2014. The CLO3403/CLO3404 two-component system of *Clostridium botulinum* E1 Beluga is important for cold shock response and growth at low temperatures. *Appl Environ Microbiol.* 80(1):399-407.

The publications are referred to in the text by their Roman numerals.

## Abbreviations

ABC	ATP binding cassette
Agr	Accessory gene regulator
ATP	Adenosine triphosphate
ATCC	American Type Culture Collection
AU	Absorption unit
BCAA	Branched-chain amino acid
BIG-IV	Human-derived botulism immune globulin intravenous
BoNT	Botulinum neurotoxin
bp	Base pair
BSA	Bovine serum albumin
cDNA	Complementary DNA
CDS	Coding sequence
CTP	Cytidine triphosphate
dI-dC	deoxyinosinic-deoxycytidylic
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
<i>e.g.</i>	<i>exempli gratia</i>
ELISA	Enzyme-linked immunosorbent assay
EMSA	Electrophoretic mobility shift assay
FDR	False discovery rate
GTP	Guanosine triphosphate
HA	Haemagglutinin
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
IPTG	Isopropyl $\beta$ -D-1-thiogalactopyranoside
kDa	Kilodalton
LB	Lysogeny broth
NTNH	Nontoxic-nonhaemagglutinin protein
OD <sub>600</sub>	Optical density at 600 nm
Orf	Open reading frame
PCR	Polymerase chain reaction
qPCR	Quantitative real-time PCR
REFED	Refrigerated processed food of extended durability
RNA	Ribonucleic acid
<i>rrn</i>	Ribosomal RNA
RT	Reverse transcription
RT-qPCR	Quantitative real-time reverse transcription PCR
SASP	Small acid-soluble protein
SDS-PAGE	Sodium dodecyl sulphate–polyacrylamide gel electrophoresis
SNAP-25	Synaptosomal-associated protein of 25 kDa
SNARE	Soluble N-ethylmaleimide-sensitive-factor attachment receptor
TCS	Two-component signal transduction system

TPGY	Tryptone-peptone-glucose-yeast extract
tRNA	Transfer RNA
TTP	Thymidine triphosphate
UV	Ultraviolet
VAMP	Vesicle-associated membrane protein
WT	Wild type

# 1 Introduction

*Clostridium botulinum* is a Gram-positive, strictly anaerobic, rod-shaped bacterium that forms highly resistant endospores and produces the extremely potent botulinum neurotoxin (BoNT). Intoxication with BoNT, most commonly through the consumption of contaminated food, causes flaccid paralysis known as botulism (Peck, 2009). Foodborne botulism has probably provoked human death from the beginning of mankind, but detailed descriptions of the disease were first presented at the beginning of the 19<sup>th</sup> century in southwestern Germany, when fatal food poisoning cases following the consumption of smoked blood sausages increased (Erbguth and Naumann, 1999). “Sausage poisoning” attracted attention in the Kingdom of Württemberg, and Justinus Kerner (1786–1862), a poet and medical officer in the small town of Welzheim, started to investigate the mysterious disease. In 1820, Kerner published the first detailed description of botulism symptoms. Two years later, he published more case reports and hypotheses about the “sausage poison”, which he now named “fat poison” or “fatty acid” in: “Das Fettgift oder die Fettsäure und ihre Wirkungen auf den thierischen Organismus, ein Beytrag zur Untersuchung des in verdorbenen Würsten giftig wirkenden Stoffes” [“The fat poison or the fatty acid and its effects on the animal body system, a contribution to the examination of the substance responsible for the toxicity of bad sausages”] (Erbguth and Naumann, 1999; Erbguth, 2004; Erbguth, 2008). Kerner concluded that the toxin is a biological substance that develops under anaerobic conditions and interrupts the capacity of nerve conduction. Finally, Kerner suggested that small doses of the toxin could be used for the treatment of diseases that originate from hyperexcitation of the nervous system. Kerner became the expert on sausage poisoning, which was then also known as “Kerner’s disease”, before it was named botulism (from the Latin *botulus* meaning sausage) (Torrens, 1998; Erbguth, 2004). However, the discovery that a bacterium produces the toxin causing botulism occurred some decades later. After a botulism outbreak at a funeral meal in the small Belgian village of Ellezelles in 1895, the case-investigating microbiologist, Emile Pierre Marie van Ermengem (1851–1922) of the University of Ghent, was able to isolate an anaerobic microorganism from the funeral meal (ham) and from the corpses of the victims. Van Ermengem characterized the anaerobic bacterium, performed animal experiments and concluded that the toxin causing botulism or sausage poisoning is produced by a specific anaerobic bacterium which he named *Bacillus botulinus* (Devriese, 1999; Erbguth, 2008). Later, the organism was renamed *Clostridium botulinum*.

After the pioneering work of Kerner and van Ermengem, research on *Clostridium botulinum* and BoNT during the last century produced a detailed understanding of how BoNT paralyzes motor neurons (Schiavo *et al.*, 1992; Pirazzini *et al.*, 2017) and enabled, as already proposed by Kerner in 1822, the development of BoNT as a therapeutic used in the treatment of several spastic muscular disorders such as torticollis, various forms of dystonia, and eye movement disorders (Johnson, 1999; Lim and Seet, 2010). Additionally, it has been shown that several non-toxic neurotoxin-associated proteins attach to the BoNT molecule to protect and assist BoNT during gastrointestinal passage in the host (Lam and Jin, 2015). The characterization of *C. botulinum* growth and neurotoxin synthesis further revealed that the production of BoNT and the non-toxic neurotoxin-associated proteins is growth phase

dependent, with peak neurotoxin gene transcription at the transition from exponential to stationary growth (Bradshaw *et al.*, 2004; Couesnon *et al.*, 2006; Chen *et al.*, 2008). In the same growth phase, *C. botulinum* starts to form highly resistant cell structures (Kirk *et al.*, 2014a) known as endospores, which facilitate the survival of the organism during harsh environmental conditions, including starvation, heat, high salt concentrations, radiation and acidity (Nicholson *et al.*, 2000). The induction of both traits when facing nutrient limitation suggests common regulation. Co-regulation of BoNT synthesis and sporulation has not been studied but might play an essential role in the life of *C. botulinum* and represents a possible intervention point for the control of this pathogen.

In order to control the risk of botulism outbreaks, a better understanding of stress response mechanisms in *C. botulinum* is important. *C. botulinum* is able to germinate, grow and produce BoNT under various stressful conditions, which might be encountered during food processing and/or food preservation (Dahlsten *et al.*, 2015; Derman *et al.*, 2015). Most notably, some *C. botulinum* strains can grow at refrigeration temperatures, which makes *C. botulinum* a serious hazard in minimally-processed, vacuum-packaged foods relying on cold storage for preservation (Lindström *et al.*, 2006; Peck, 2006). Mild heat treatments during the processing of these foods might not sufficiently eliminate all *C. botulinum* spores, and minimal storage temperature abuse might already allow outgrowth from spores with subsequent BoNT production by vegetatively growing *C. botulinum* cells. Mechanisms responsible for cold tolerance and the cold-shock response have been studied in the mesophilic Group I *C. botulinum* (Dahlsten *et al.*, 2015), but are scarcely known in the psychrotrophic Group II *C. botulinum* strains, the most hazardous strains regarding BoNT production in foods during cold storage.

## 2 Review of the literature

### 2.1 *Clostridium botulinum* and botulism

#### 2.1.1 *Clostridium botulinum*

The genus *Clostridium* belongs to the phylum Firmicutes and describes Gram-positive, anaerobic, rod-shaped, low G+C nucleotide content bacteria that form highly resistant endospores (Cato *et al.*, 1986). *Clostridium* is a very old bacterial genus, which is suggested to have appeared on Earth 2.7 billion years ago, before the great oxygenation event (Paredes *et al.*, 2005). Since then, around 100 bacterial species belonging to the genus *Clostridium* have evolved. Most of them are harmless to human and animal health, but some of them produce toxins, like *C. tetani*, *C. perfringens*, *C. difficile* and *C. botulinum* (Cato *et al.*, 1986; Hatheway, 1990). *C. botulinum* produces the extremely potent BoNT, which blocks neurotransmitter release in cholinergic nerves (Schiavo *et al.*, 1992; Blasi *et al.*, 1993), thereby causing the rare but severe neuroparalytic disease botulism, which affects both humans and animals (vertebrates). *C. botulinum* is widespread in nature, with spores inhabiting the soil and aquatic environments in many parts of the world (Peck, 2009). The species *C. botulinum* is genetically and phenotypically diverse and primarily defined by the ability to produce BoNT. Strains belonging to the species *C. botulinum* are divided into four groups (Groups I–IV) based on their physiological and phenotypic characteristics (Hatheway, 1990), which reflect the phylogenetic background of the strains (Hill *et al.*, 2007). Due to the high heterogeneity among *C. botulinum* strains, the four groups could even be classified as different species.

Each *C. botulinum* strain is assigned to the BoNT serotype(s) (A–G) produced by the strain (Hatheway, 1990). The seven well-established BoNT serotypes (A–G) are classified according to their antigenic properties, and each *C. botulinum* group produces certain BoNT serotypes. Group I and II *C. botulinum* produce BoNT types A, B, F and/or F/A (a mosaic version of F and A) and B, E or F, respectively, and are associated with human botulism. Furthermore, a new BoNT serotype (X) was recently identified in a Group I *C. botulinum* type B strain (Zhang *et al.*, 2017). Group III *C. botulinum* produces BoNT types C or D, or their mosaic versions C/D or D/C, and is primarily associated with animal botulism. BoNT type G is formed by Group IV *C. botulinum* (also known as *Clostridium argentinense*), but no reports of botulism cases related to BoNT type G are available. Additionally, some strains of the species *Clostridium butyricum* and *Clostridium baratii* are capable of forming BoNT of type E and F, respectively. To date, these strains have not been considered to belong to the species *C. botulinum*, but together with *C. botulinum* Groups I–IV form the entity of BoNT-producing clostridia (Table 1).

Table 1. Characteristics of BoNT-producing clostridia

	Group I <i>C. botulinum</i>	Group II <i>C. botulinum</i>	Group III <i>C. botulinum</i>	Group IV <i>C. botulinum</i>	<i>C. butyricum</i>	<i>C. baratii</i>
BoNT type	A, B, F, F/A, X	B, E, F	C, D, C/D, D/C	G	E	F
Location of BoNT cluster	Chromosome and/or plasmid	Chromosome or plasmid	Bacteriophage, plasmid	Plasmid	ND <sup>a</sup>	ND
Growth temperature range	10–48 °C	3–45 °C	15 °C–ND	12–45 °C	10–40 °C	20 °C–ND
Optimum growth temperature	37–42 °C	26–37 °C	40 °C	37 °C	30–37 °C	30–40 °C
Minimum pH for growth	4.6	5.0	5.1	ND	4.8	ND
Growth-inhibiting NaCl concentration	10%	5%	3%	>3%	5%	5%
Proteolytic activity (casein digestion)	+	-	+/-	+	-	-
Lipase production	+	+	+	-	-	-
Carbohydrate fermentation	+	+	+	-	+	+
Spore heat resistance <sup>b</sup>	D <sub>121 °C</sub> = 0.21 min	D <sub>82 °C</sub> = 2.4 min	D <sub>104 °C</sub> = 0.9 min	D <sub>104 °C</sub> = 1.1 min	D <sub>100 °C</sub> < 0.1 min	ND

Source: (Hatheway, 1990; Lindström and Korkeala, 2006; Hinderink *et al.*, 2009; Peck, 2009; Derman *et al.*, 2011; Dover *et al.*, 2013; Johnson, 2013; Zhang *et al.*, 2013a; Hosomi *et al.*, 2014; Zhang *et al.*, 2017)

<sup>a</sup> ND: no data available; <sup>b</sup> D: time for 10-fold reduction in the viable spore count at a certain temperature in phosphate buffer (pH 7)



## 2.1.2 Botulism

Botulism is a rare but severe neuroparalytic disease (mortality rate 3–5% [Leclair *et al.*, 2013b; Jackson *et al.*, 2015]) caused by the action of BoNT. BoNT cleaves soluble N-ethylmaleimide-sensitive-factor attachment receptor (SNARE) proteins in presynaptic nerve endings and prevents the release of the neurotransmitter acetylcholine into the neuromuscular junction, thereby causing flaccid paralysis (Pirazzini *et al.*, 2017). Initial symptoms of botulism include blurred or double vision, difficulties in speaking and swallowing, dry mouth and facial paralysis. Descending paralysis affects the limbs, gastrointestinal tract, bladder and respiratory muscles. Patients suffering from botulism are usually fully conscious. When untreated or wrongly diagnosed, botulism might cause lethality due to respiratory failure. After the diagnosis of botulism, patients are treated with antitoxin to neutralize the circulating toxin in the blood stream, and assistance in ventilation is frequently needed to ensure respiratory function (Sobel, 2005; Erbguth, 2008). Mechanical respiration during hospitalization can be needed for up to months depending on the severity of the disease. Full recovery of patients may take several months and begins with the sprouting of new endplates in the affected parental axons. Later, the activity of the parental neuronal junction becomes restored and the formed sprouts disappear (Meunier *et al.*, 2003).

Different forms of botulism are classified depending on how the toxin enters the patient. Botulism is either assigned as an intoxication, most commonly through the consumption of preformed toxin in food or drink (foodborne botulism), or as a toxicoinfection due to BoNT production by vegetatively growing *C. botulinum* cultures in the intestine (intestinal botulism) or in deep anaerobic wounds (wound botulism).

### *Foodborne botulism*

Foodborne botulism is the classical form of botulism and relies on the ingestion of foods contaminated with BoNT. *C. botulinum* spores surviving food processing may germinate in foods under suitable growth conditions, including anaerobiosis and a growth-supporting storage temperature. During vegetative growth, BoNT is formed and released by the cells. Foods frequently related to foodborne botulism include home-prepared canned or bottled meat and vegetable products, traditionally-prepared fish or marine mammals, and commercially available mildly treated or improperly processed packaged food products (Lindström *et al.*, 2006; Peck, 2009; Leclair *et al.*, 2013b; Carter and Peck, 2015). Food treatments favouring the outgrowth of *C. botulinum* into toxic cultures include mild pasteurization, anaerobic packaging and extended storage at an inadequate temperature (Lindström *et al.*, 2006; Peck, 2006). *C. botulinum* spores are widespread in nature and contamination of any raw food material cannot be entirely excluded. Therefore, food processing treatments eliminating contamination and/or preventing spore germination have to be applied in order to avoid foodborne botulism cases.

### *Intestinal and infant botulism*

Under certain circumstances, ingested spores may germinate and create a toxic culture in the intestinal tract. Intestinal botulism in adults relies on intestinal abnormalities and represents a rare form of botulism. Generally, a healthy gut microbiota does not allow colonization with *C. botulinum*. Decreased microbial competition in the gut, for example caused by intestinal diseases such as Crohn's disease or by antibiotic administration after surgery, enables the germination and growth of *C. botulinum* in the gut (McCroskey and Hatheway, 1988; Griffin *et al.*, 1997).

Intestinal botulism also occurs in infants younger than one year old due to the lack of a competitive intestinal microbiota and is then called infant botulism (Fenicia and Anniballi, 2009). Infant botulism is the most common form of botulism in the US (Jackson *et al.*, 2015). After outgrowth from ingested spores, *C. botulinum* is able to colonize the large intestine of infants and BoNT is formed *in vivo* (Mills and Arnon, 1987). Most commonly, the source of ingested *C. botulinum* spores is honey or dust (Nevas *et al.*, 2002; Nevas *et al.*, 2005; Derman *et al.*, 2014), but pet turtles have also been associated with infant botulism (Shelley *et al.*, 2015). Typical symptoms of infant botulism are constipation, poor feeding, lethargy, a weak cry, hypotonia, dilated pupils and missing reflexes. After diagnosis, patients receive extensive supportive care and the human-derived botulism immune globulin intravenous (BIG-IV) may be administered (Arnon *et al.*, 2006). Recovered infants pose a threat to others, as shedding of BoNT and/or *C. botulinum* may continue for several months (Derman *et al.*, 2014).

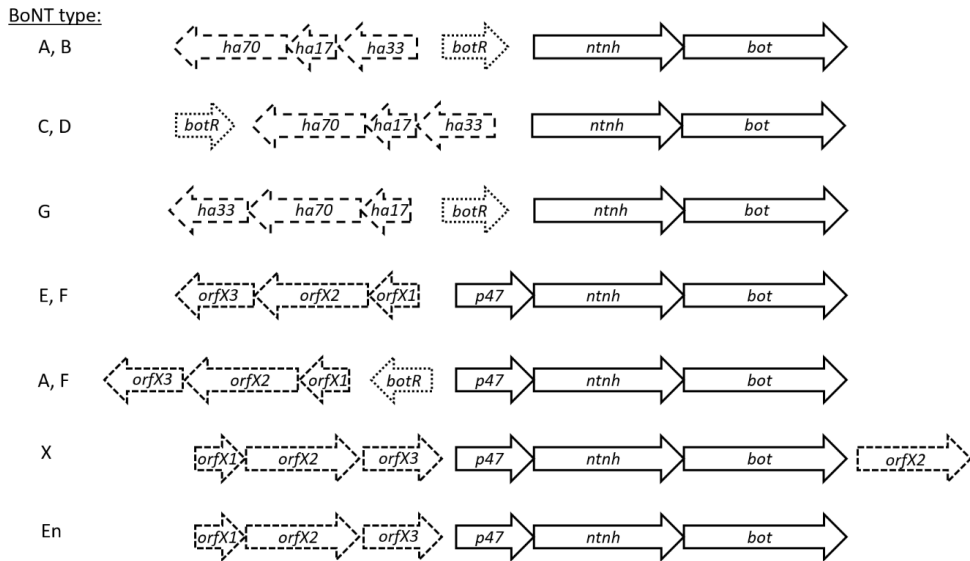
### *Wound botulism*

Similarly to the closely related pathogen *C. tetani*, which produces tetanus neurotoxin causing muscle spasms, *C. botulinum* can colonize and grow in deep anaerobic wounds. After outgrowth from spores and toxin release by subsequent vegetatively growing *C. botulinum* cells in wounds, BoNT enters the blood stream and causes symptoms similar to those observed in foodborne botulism, with the exception of negligible effects on the gastrointestinal tract. Wound botulism most commonly results from the contamination of traumatic injuries (Peck, 2009). However, in the recent decades, an increased number of wound botulism cases in injecting drug users has been reported (Werner *et al.*, 2000; Akbulut *et al.*, 2005; Schroeter *et al.*, 2009). Contaminated heroin or unsterile needles provide a suitable transmission vehicle for spores into anaerobiosis when drugs are injected subcutaneously or intramuscularly. Like other forms of botulism, wound botulism is treated by antitoxin administration, but proper cleaning of the infected wound and antibiotic administration is additionally required to eliminate *C. botulinum* cells and spores in the patient's body (Sobel, 2005).

## 2.2 Botulinum neurotoxin (BoNT)

### 2.2.1 BoNT gene cluster

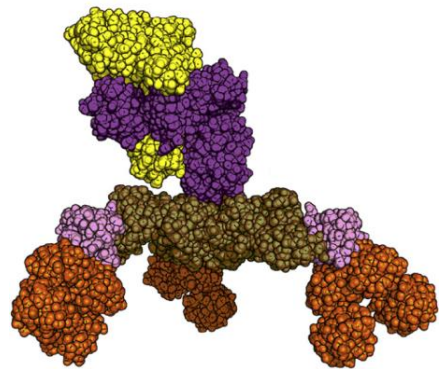
The BoNT gene (*bot*) and the genes encoding the neurotoxin-associated proteins form the BoNT gene cluster (Fig. 1), which can reside on chromosomes, plasmids or bacteriophages (Hill and Smith, 2013; Zhang *et al.*, 2013a; Sakaguchi *et al.*, 2015). Generally, the BoNT gene clusters consist of two operons (the *bot* operon and either the *ha* operon or the *orfX* operon) transcribed in opposite directions. Some BoNT gene clusters additionally contain the *botR* gene, which resides between the two operons or downstream of the *ha* operon (Hill and Smith, 2013). Two main arrangements of the BoNT gene cluster exist, either encoding the *ha* operon (*ha33*, *ha17*, and *ha70*) or the *orfX* operon (*orfX1-3*). The haemagglutinins (HA33, HA17, and HA70) encoded by the *ha* operon help BoNT in translocation across the intestinal epithelium (Amatsu *et al.*, 2013; Fujinaga *et al.*, 2013), whereas the expression and function of the OrfX1-3 is not well understood. The BoNT gene clusters encoding the *ha* operon always contain *botR*, which encodes the alternative sigma factor BotR. BotR activates transcription of the *ha* operon and the *bot* operon by directing the RNA polymerase to the core promoters of the two operons (Marvaud *et al.*, 1998b). The *bot* operon of BoNT clusters encoding the *ha* operon consists of *bot* and *ntnh*, encoding the nontoxic-nonhaemagglutinin protein NTNH which protects BoNT against gastric degradation and possibly other harsh environments (Gu *et al.*, 2012). BoNT clusters encoding the *orfX* operon contain a *bot* operon that, in addition to *ntnh* and *bot*, also harbours *p47*, a gene encoding a lipid-binding protein of unknown function (Gustafsson *et al.*, 2017; Lam *et al.*, 2017). Additionally, this type of BoNT cluster may or may not contain *botR* between the two operons. In general, the *ha* toxin gene clusters harbour *bot* genes of type A, B, C, D or G, whereas the *orfX* toxin gene clusters harbour *bot* genes of type E, F, X or certain A subtypes (Hill and Smith, 2013; Zhang *et al.*, 2017). As an exception to the described arrangements of BoNT clusters, the *orfX* operon of the newly identified BoNT/X cluster is transcribed in the same direction as the *bot* operon and an additional copy of *orfX2* is present downstream of *botX* (Zhang *et al.*, 2017). A similarly arranged BoNT cluster encoding a novel BoNT type (BoNT/En) was identified in a bacterial species outside of *Clostridium*, namely *Enterococcus faecium* (Zhang *et al.*, 2018). The extreme diversity of BoNT gene clusters and the BoNT types encoded by each type of toxin gene cluster are illustrated in Figure 1. Moreover, genes encoding the BoNT-like toxins BoNT/Wo and Cp1 toxin have been identified in *Weissella oryzae* (Mansfield *et al.*, 2015) and *Chryseobacterium piperi* (Mansfield *et al.*, 2017), respectively. However, these BoNT-like toxins, especially the Cp1 toxin, are quite distinct from other BoNTs, and the respective toxin gene clusters only contain *ntnh*- and *bot*-like genes and no *ha/orfX/p47* genes.



**Figure 1. Simplified illustration of BoNT gene cluster arrangements. Genes are indicated with arrows. Arrows next to each other with the same outline style represent operons.**

## 2.2.2 Structure of the BoNT complex

BoNTs are produced as protein complexes together with the nontoxic-nonhaemagglutinin protein (NTNH) and three haemagglutinins (HA70, HA33, and HA17), or with NTNH and three proteins of unknown function (OrfX1-3) (Kalb *et al.*, 2017). The BoNT-NTNH-HA complex (see Fig. 2) has a bacteriophage-like appearance consisting of an ovoid-shaped BoNT-NTNH entity standing on a triskelion-shaped HA structure formed by three HA70, three HA17 and six HA33 protein subunits (Lam and Jin, 2015). BoNT and NTNH, which does not have any toxic activity but resembles the structure of BoNT, form a heterodimer by enwinding each other (Gu *et al.*, 2012). NTNH thereby protects the BoNT molecule from degradation (proteolytic enzymes) and other harm (*e.g.* low pH) during its travel through the digestive tract (Gu *et al.*, 2012). The HA component of the BoNT complex interacts with glycoprotein 2 expressed on intestinal microfold cells and facilitates the transport of the BoNT complex across the intestinal epithelium (Matsumura *et al.*, 2015; Fujinaga and



**Figure 2. BoNT complex structure. The BoNT complex consists of BoNT (yellow), NTNH (purple), 3 HA70 (sand), 3 HA17 (pink) and 6 HA33 (orange). Image modified from Lam and Jin (2015) and reprinted with the permission of Elsevier.**

Popoff, 2017). Additionally, it has been shown that HA interacts with E-cadherin, which results in the disruption of intercellular junctions in the intestinal epithelium and mediates BoNT translocation via the paracellular route (Matsumura *et al.*, 2008; Sugawara *et al.*, 2010; Fujinaga *et al.*, 2013; Fujinaga and Popoff, 2017). After translocation, BoNT is released into the blood stream and travels to presynaptic motor neurons.

Recently, it was shown that the OrfX proteins are part of the BoNT complex in *C. botulinum* strains encoding the *orfX* toxin gene cluster (Kalb *et al.*, 2017) and that the OrfX and P47 proteins, in contrast to the HA proteins, bind lipids (Gustafsson *et al.*, 2017; Lam *et al.*, 2017). However, their role in intestinal absorption warrants further research, and an alternative role of the OrfX and P47 proteins in the secretion of BoNT across the *C. botulinum* cell membrane has been suggested (Lam *et al.*, 2017).

### 2.2.3 Activation and enzymatic activity of BoNT

The BoNT molecule is initially produced as a single-chain polypeptide (150 kDa). Proteolytic cleavage, also known as “nicking”, of the single-chain toxin molecule results in the formation of a dichain molecule consisting of a heavy-chain molecule (100 kDa) and a light-chain molecule (50 kDa), which are connected by a single disulphide bond (Sathyamoorthy and DasGupta, 1985; Dekleva and Dasgupta, 1990). Proteolytic cleavage of the initial BoNT molecule is required to confer neurotoxic activity. In Group I *C. botulinum*, endogenous proteases activate BoNT, whereas in non-proteolytic Group II *C. botulinum*, external proteases produced in the gut are responsible for BoNT activation (Simpson and Dasgupta, 1983; Sathyamoorthy and DasGupta, 1985; Dekleva and DasGupta, 1989). Once the BoNT reaches cholinergic nerve endings, the C-terminal binding domain of the heavy chain interacts with two receptors, a ganglioside and a glycosylated synaptic vesicle protein, of the presynaptic plasma membrane, and BoNT is subsequently internalized by endocytosis (Montecucco, 1986; Rummel, 2013; Pirazzini *et al.*, 2017). The acidic environment in endosomes causes conformational changes of BoNT, and the N-terminal translocation domain of the heavy chain facilitates translocation of the unfolded light chain across the endosomal membrane (Keller *et al.*, 2004; Pirazzini *et al.*, 2011). The light chain is released into the cytosol by cleavage of the disulphide bond. After folding of the light chain in the cytosol, the molecule acquires its zinc-dependent metalloprotease activity (Fischer and Montal, 2007). The light chain specifically cleaves soluble N-ethylmaleimide-sensitive-factor attachment receptor (SNARE) proteins in presynaptic nerve endings (Schiavo *et al.*, 2000). SNARE proteins are essential for the fusion of neurotransmitter-carrying vesicles with presynaptic membranes of motor neurons. Therefore, cleavage of SNARE proteins prevents neurotransmitter release into the neuromuscular junction and causes flaccid paralysis. The light chains of different BoNT serotypes cleave specific SNARE proteins. BoNT types A, C and E cleave a synaptosomal-associated protein of 25 kDa (SNAP-25) and BoNT types B, D, F, G, Wo and X cut synaptobrevins, vesicle-associated membrane proteins (VAMPs). BoNT/C additionally cuts syntaxin, another SNARE protein, and BoNT/En cuts SNAP-25 and VAMP. Moreover, the

cleavage site within the SNARE proteins is serotype specific (Zornetta *et al.*, 2016; Pirazzini *et al.*, 2017; Zhang *et al.*, 2017; Zhang *et al.*, 2018).

## 2.3 Regulation of BoNT synthesis

### 2.3.1 BoNT synthesis is growth phase dependent

In Group I *C. botulinum* type A and Group II *C. botulinum* type E batch cultures, transcription of the neurotoxin gene cluster operons starts slowly at the beginning of exponential growth, increases rapidly throughout exponential growth, and peaks during the transition from exponential to stationary growth when nutrients become limited. During stationary growth, the expression of the neurotoxin genes declines strongly (Bradshaw *et al.*, 2004; Couesnon *et al.*, 2006; Chen *et al.*, 2008). Accordingly, the concentration of BoNT molecules increases until stationary growth and remains stable thereafter. Whereas BoNT was found to be intracellular during exponential growth, increased release of BoNT into the extracellular environment was observed during stationary growth (Bradshaw *et al.*, 2004; Couesnon *et al.*, 2006). However, the mechanism of BoNT release is unknown, but might be associated with vegetative cell lysis and/or sporulation-dependent mother cell lysis during stationary growth. Altogether, transcriptional regulation of BoNT synthesis appears to be growth phase dependent in *C. botulinum*, with peak toxin gene expression at the transition from exponential to stationary growth.

### 2.3.2 Transcriptional regulation of BoNT synthesis

#### *BotR*

The *botR* gene is part of the BoNT gene cluster and encodes the alternative sigma factor BotR, which directs the RNA polymerase to the core promoters of the neurotoxin gene operon and the *ha* operon (Marvaud *et al.*, 1998b). BotR specifically recognizes and binds the -10 (GTTATA) and -35 (TTTACA) sequences of the respective promoters. BotR-directed neurotoxin gene expression is probably conserved in all *C. botulinum* type A, B, C, D and G strains, and in Group I *C. botulinum* type F strains. However, genomes of Group II *C. botulinum* type E and F lack the *botR* gene (Hill and Smith, 2013).

BotR is homologous to the clostridial toxin regulators TetR of *C. tetani*, TcdR of *C. difficile* and UviA of *C. perfringens*. These alternative sigma factors form a subgroup of the sigma factor 70 family. TetR, the closest homolog to BotR, activates tetanus toxin gene expression in *C. tetani* (Marvaud *et al.*, 1998a) and TcdR positively regulates the expression of toxins A and B in *C. difficile* (Mani and Dupuy, 2001). UviA activates the expression of a UV-inducible bacteriocin gene in *C. perfringens* (Garnier and Cole, 1988; Dupuy *et al.*,

2005) and possibly regulates *bot* transcription in BoNT/F-producing *C. baratii*, which lacks *botR* (Dover *et al.*, 2014).

### *Two-component signal transduction systems*

In order to interact with the extracellular environment, bacteria have to sense their environment and transform the gathered stimuli into adequate responses. Two-component signal transduction systems (TCSs) represent an important “sensing-signalling” mechanism in bacteria and facilitate the appropriate response to many different environmental conditions (Krell *et al.*, 2010). A typical TCS consists of a sensory histidine kinase located in the plasma membrane and a DNA-binding response regulator present in the cytoplasm of the bacterial cell. The histidine kinase senses the designated stimulus with the N-terminal sensor domain and phosphorylates the N-terminal receiver domain of the cognate response regulator (Hoch, 2000). Phosphorylation of the N-terminal receiver domain causes conformational changes and activates the DNA-binding capacity of the C-terminal output domain. Binding of the response regulator to specific target DNA regulates the transcription of selected genes to facilitate an appropriate genetic response to the stimulus (West and Stock, 2001; Gao and Stock, 2009). Several pathogenic bacteria use TCSs to regulate virulence factors in response to changing growth conditions (Beier and Gross, 2006).

In *C. botulinum*, several TCSs have been shown to be involved in neurotoxin gene regulation (Connan and Popoff, 2015). The TCSs CLC\_1093/CLC\_1094, CLC\_1914/CLC\_1913 and CLC\_0661/CLC\_0663 positively regulate BoNT/A synthesis in *C. botulinum* type A Hall (Connan *et al.*, 2012). Indeed, in strains with antisense-RNA silenced translation of the respective TCSs, BoNT/A synthesis was significantly reduced compared to the wild-type strain. However, reduced BoNT/A expression was not caused by impaired *botR* transcription in the mutant strains, suggesting that the TCSs regulate (indirectly or directly) *botA* transcription independently of the alternative sigma factor BotR. The signals activating these TCSs are unknown and the interplay of these TCSs with other neurotoxin regulators remains to be elucidated.

Recently, a TCS (CBO0787/CBO0786) negatively regulating BoNT/A synthesis was identified, which represents the only known transcriptional repressor of BoNT synthesis (Zhang *et al.*, 2013). The response regulator CBO0786 binds specifically to the -10 binding region of BotR in promoters of the *botA* and *ha* operons, and blocks binding of the RNA polymerase-BotR complex, thereby preventing transcription of the *botA* and *ha* operons. Repression of *botR* transcription by CBO0786 is unlikely, as no CBO0786 binding sites were detected upstream of *botR*. The signal triggering the histidine kinase CBO0787 was not identified and remains to be elucidated in order to better understand the environmental conditions repressing BoNT synthesis.

## Quorum sensing

Quorum sensing allows bacteria to adapt in response to high cell density within a population (Miller and Bassler, 2001; Waters and Bassler, 2005). A quorum sensing system is based on an autoinducer that is released by the cells. The concentration of the autoinducer increases when the cell number increases, and after reaching a certain threshold level in the culture, the autoinducer triggers a signalling cascade that finally regulates cell-density-dependent gene expression. Quorum sensing controls many important bacterial traits such as biofilm formation, bioluminescence, competence, sporulation and virulence. In the Gram-positive pathogen *Staphylococcus aureus*, the accessory gene regulator (Agr) quorum sensing system consisting of the AgrBDCA proteins responds to high cell density by upregulation of virulence proteins (Novick and Geisinger, 2008; Wang and Muir, 2016). AgrD is the precursor of the autoinducing peptide, which is processed and transported to the extracellular space by the membrane protein AgrB. Increasing concentrations of the autoinducing peptide activate the membrane-embedded histidine kinase AgrC, which transfers the signal to the response regulator AgrA. Thereafter, AgrA induces the expression of RNAIII, a regulatory RNA, which controls the expression of virulence factors.

In *C. botulinum*, it has been shown that genes encoding homologues of the AgrB and AgrD proteins are present in two distinct *agrBD* loci. To characterize the role of the two autoinducing peptide precursor proteins AgrD1 and AgrD2, insertional inactivation of the respective genes was conducted (Cooksley *et al.*, 2010). Phenotypic analysis of *agrD1* and *agrD2* mutants revealed a 1000-fold and 70-fold reduction in spore numbers, respectively, compared with *C. botulinum* ATCC 3502 wild type. Furthermore, BoNT synthesis was delayed but reached the wild-type levels of BoNT/A in *agrD1* mutant cultures, and *agrD2* mutant cultures showed reduced BoNT synthesis by producing approximately one third of the BoNT amount produced by wild-type cultures. Hence, the results suggest that *agrBD2* is more important for BoNT synthesis than *agrBD1*, which appears to play a more prominent role in the control of sporulation (Cooksley *et al.*, 2010). Genes encoding homologues of the TCS AgrC/AgrA could not be detected in *C. botulinum* genomes. Whether one or more of the known BoNT-regulating TCS responds to increased levels of the autoinducing peptides encoded by *agrD1* and *agrD2* remains to be proven. To sum up, it appears that BoNT synthesis and sporulation are influenced by quorum sensing in *C. botulinum* ATCC 3502.

## CodY

The transition-state regulator CodY of low-G+C Gram-positive bacteria regulates metabolism in order to adapt bacterial cells for the transition from exponential growth to stationary growth. In *B. subtilis*, CodY reacts to changing intracellular levels of GTP and branched chain amino acids (BCAAs) and regulates the expression of over 100 genes (Ratnayake-Lecamwasam *et al.*, 2001; Molle *et al.*, 2003b; Sonenshein, 2007). During the transition from exponential to stationary growth, intracellular levels of GTP and BCAA decrease. This leads to the de-repression of CodY-repressed genes, which are mostly related



to amino acid synthesis and the transport of amino acids, peptides and sugars. Additionally, CodY activates the fermentation of glycolysis-derived pyruvate into solvents during carbon overflow (Sonenshein, 2007). CodY is highly conserved in low-G+C Gram-positive bacteria and also known for the nutrition-related regulation of virulence factors in many low-G+C Gram-positive pathogens, including *C. difficile* (Dineen *et al.*, 2007), *C. perfringens* (Li *et al.*, 2013), *B. cereus* (Frenzel *et al.*, 2012), *L. monocytogenes* (Lobel *et al.*, 2012) and *S. aureus* (Majerczyk *et al.*, 2010).

In *C. botulinum*, BoNT synthesis is influenced by the availability of certain carbohydrates and amino acids (Bonventre and Kempe, 1959; Patterson-Curtis and Johnson, 1989; Leyer and Johnson, 1990; Fredrick *et al.*, 2017) and CodY-dependent nutrition status-related BoNT regulation has recently been demonstrated (Zhang *et al.*, 2014). Clostron-mediated inactivation of CodY resulted in significantly decreased BoNT/A levels compared with the wild-type *C. botulinum* ATCC 3502 strain, and overexpression of CodY significantly increased BoNT/A levels. Furthermore, it was shown that CodY directly interacts with the *bot* promoter and that GTP, an indicator of the nutritional status, increases the binding affinity of CodY to the *bot* promoter. CodY binds specifically to a putative CodY binding site upstream of the transcriptional start site and immediately downstream of the -10 site of the core promoter, which is recognized by BotR. It was suggested that CodY possibly enhances the binding of BotR and/or the RNA polymerase to the core promoter and thereby induces *bot* transcription.

A computational study (Ihekwaba *et al.*, 2016), however, suggested that CodY represses transcription from the *ntnh/botA* promoter and induces BoNT synthesis by repressing the transcription of the neurotoxin repressor *cho0786* (two putative CodY binding sites upstream of *cho0786*) and by activating *botR* transcription (one putative CodY binding site upstream of *botR*). In their computational model of toxigenesis in *C. botulinum* type A1, the authors suggest that CodY has two nutrition-level-dependent roles in controlling BoNT synthesis: (i) when nutrient availability is high, CodY represses the *ntnh/botA* operon and (ii) when nutrients become limited, CodY activates BoNT synthesis by repressing the transcription of the negative regulator *cho0786* and by inducing the transcription of *botR*. The mechanism behind these putative different behaviours of CodY is unknown and could be dependent on co-factors, certain nutrients, metabolic products (for example GTP, which increased the binding affinity of CodY to the *ntnh/botA* promoter), interaction with other regulating proteins or CodY binding site positions.

## 2.4 Sporulation

### 2.4.1 Endospores

Bacilli and clostridia form highly resistant endospores in order to survive unfavourable environmental conditions. Heat-resistant spores were discovered and described by Ferdinand Cohn and Robert Koch in the 1870s (Drews, 2000; Gould, 2006), thereby also

contributing to the disproof of spontaneous generation as the origin of organisms. Bacterial spores are the most resistant cell type known and are extremely resistant to heat, desiccation, radiation, chemicals, pressure, extreme pH, digestive enzymes and other stress factors (Nicholson *et al.*, 2000). They can be dormant for a very long period of time without detectable metabolic activity (Setlow, 2007). It was even proposed that spores that may have been formed millions of years ago are still able to germinate (Cano and Borucki, 1995; Vreeland *et al.*, 2000).

### **2.4.2 Endospore structure**

Endospores are differently shaped, varying from spherical to elliptical in appearance, and are formed centrally, subterminally or terminally within mother cells (Saxena and Awasthi, 2003). In some species, such as *Clostridium oceanicum*, vegetative cells produce two spores, one in each end of the rod-shaped cell (Smith, 1970). However, the morphology of spores is well conserved. A typical bacterial endospore consists of a dehydrated DNA-harboring core, which is surrounded by an inner membrane, a cortex layer, a coat, and in some species an exosporium (Setlow, 2007). The inner part of the spore, the spore core, is dehydrated and filled with dipicolinic acid associated with divalent cations, mostly  $\text{Ca}^{2+}$ , which partly protects the DNA. Protection of the spore DNA is accomplished by small acid-soluble proteins (SASPs), which bind to the DNA and protect it from heat, chemicals and UV radiation (Fairhead *et al.*, 1993; Setlow, 2006; Setlow, 2007). SASPs are produced during sporulation and are degraded during germination, providing amino acids for protein synthesis and energy metabolism for the outgrowing spore. The spore core is surrounded by a low-permeable inner membrane (actually the forespore cell membrane) containing the germination receptors (Korza and Setlow, 2013). A peptidoglycan layer known as the germ cell wall covers the inner membrane and becomes the cell wall of an outgrowing spore. The germ cell wall is surrounded by a thick cortex layer, which confers wet heat resistance. The cortex layer is built up from modified peptidoglycan (muramic acid- $\delta$ -lactam and muramic acid linked to alanine) and surrounded by the outer membrane and the spore coat (Setlow, 2007). The spore coat mainly consists of proteins and is structured into two insoluble layers (inner and outer coat). Protective enzymes are embedded in the coat and confer resistance to lytic enzymes and reactive chemicals (Henriques and Moran, 2007). In many spore-forming bacteria, the spore coat is surrounded by an additional layer, the exosporium. Exosporia vary in shape and form between species and consist of proteins including glycoproteins and enzymes. Exosporia represent the outermost part of spores and are thought to play a role in pathogenesis through adherence and spore–host interaction (Stewart, 2015).

### **2.4.3 Stages of sporulation**

The process of sporulation is highly conserved in bacilli and clostridia and subdivided into 7 morphological stages (I–VII), which are illustrated in Figure 3. In stage I, the DNA

replicates and forms axial filaments, and cells might show a cigar-shaped and swollen phenotype known as the clostridial form. This cell morphology change is unique to *Clostridium*. However, stage I of sporulation is not easy to distinguish from vegetative cells, which are often designated as stage 0. Sporulating cells become visually distinct in stage II, when asymmetric cell division separates the cell into a larger mother cell compartment and a smaller forespore compartment. During stage III, the mother cell engulfs the forespore. After forespore engulfment, sporulation becomes irreversible. Stage IV is characterized by the formation of a thick peptidoglycan layer, also known as the cortex, around the forespore, and during stage V, a spore coat consisting of various proteins covers the cortex. In stage VI, the cortex and coat mature. Sporulation ends in stage VII with the release of a mature spore by mother cell lysis (Hilbert and Piggot, 2004; Tan and Ramamurthi, 2014; Al-Hinai *et al.*, 2015).

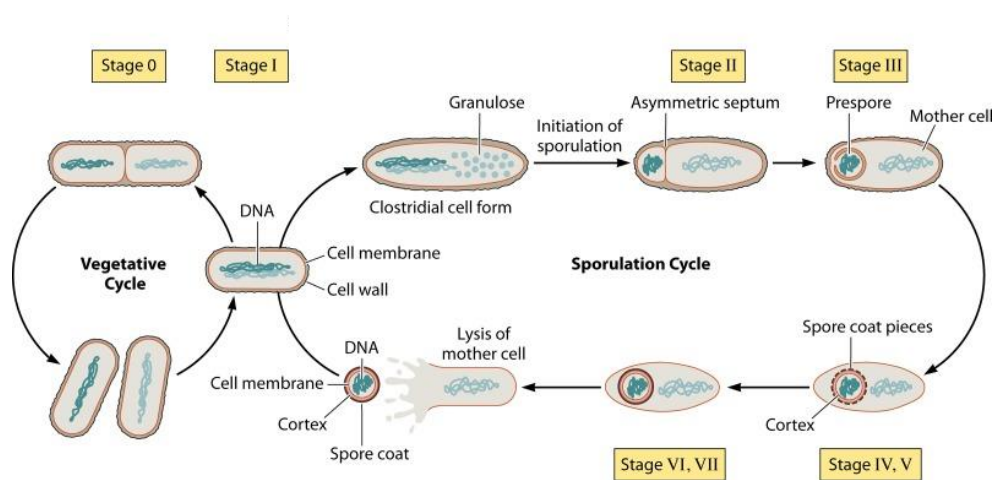


Figure 3. The morphological stages (I–VII) during sporulation in clostridia. Image modified from Al-Hinai *et al.* (2015) and reprinted with the permission of the American Society for Microbiology.

## 2.4.4 Regulation of sporulation

### *Initiation of sporulation*

The molecular mechanisms behind sporulation have been extensively studied in *B. subtilis*. In *B. subtilis*, sporulation is initiated by nutrient starvation and a high cell density (Sonenshein, 2000), whereas DNA damage prevents the initiation of sporulation (Ireton and Grossman, 1994). On the molecular level, sporulation is initiated upon the phosphorylation of the master regulator of sporulation, Spo0A (Hoch, 1993; Piggot and Hilbert, 2004). In bacilli, a phosphorelay system phosphorylates Spo0A (Spo0A~P) and activates the

sporulation pathway. The membrane-embedded orphan histidine kinases KinA and KinB detect the sporulation signal, autophosphorylate and transfer the phosphoryl group to Spo0F, which transfers the phosphoryl group via Spo0B to Spo0A. Alternatively, KinC can phosphorylate Spo0A directly. Phosphorylation of Spo0A is reversible through Spo0E, which can actively dephosphorylate Spo0A and thereby cancel the decision to sporulate. Once phosphorylated, the DNA-binding transcriptional regulator Spo0A undergoes a conformational change leading to homodimer formation with elevated DNA-binding affinity (Lewis *et al.*, 2002). Spo0A~P recognizes and binds to specific parts of the DNA, so-called Spo0A-binding boxes (TGTCGAA), and activates or represses gene transcription. To initiate sporulation, low levels of Spo0A~P are sufficient to repress the expression of the sporulation repressor AbrB (Hoch, 1993; Fujita *et al.*, 2005). Amongst other sporulation-related proteins, AbrB represses SigH, which is needed for efficient transcription of *spo0A* (Haldenwang, 1995). Therefore, Spo0A~P indirectly increases its own expression and Spo0A~P accumulates in the cells. Thereafter, Spo0A~P and SigH activate the expression of SigF, the first sigma factor of the sporulation cascade (Wu *et al.*, 1989), and SigE, the first mother-cell-specific sigma factor (Hilbert and Piggot, 2004). However, the Spo0A regulon covers more than 100 genes, which beside sporulation also play a role in various other traits, including solventogenesis, motility, metabolism and virulence (Ravagnani *et al.*, 2000; Molle *et al.*, 2003a; Pettit *et al.*, 2014). Spo0A is a global regulator preparing bacteria for post-exponential growth, most notably by initiating sporulation.

### *Sigma factor cascade*

After the initiation of sporulation by Spo0A, four sporulation-specific sigma factors (SigF, SigE, SigG and SigK) guide the sporulation process (Hilbert and Piggot, 2004). SigF in the forespore and SigE in the mother cell are active early during sporulation, whereas late-stage sporulation is controlled by SigG in the forespore and SigK in the mother cell.

The first sigma factor of the sporulation cascade, SigF, is under the control of Spo0A and SigH (Wu *et al.*, 1989). SigF becomes active in the forespore during stage II of sporulation, when sporulating cells form the asymmetric septum. Importantly, SigF controls *spoIIR*, *spoIIQ* and *sigG*. SpoIIR is membrane embedded and needed for the activation of SigE (Karow *et al.*, 1995), the early mother-cell-specific sigma factor and the next sigma factor of the sporulation cascade. SpoIIQ is part of the essential cross-membrane channel feeding the forespore (Camp and Losick, 2009). SigE, whose transcription is also induced by Spo0A, is indirectly activated by SigF and regulates gene expression during early sporulation in the mother cell (Hilbert and Piggot, 2004). SigF is needed to transcribe *spoIIIA* and *sigK*. SpoIIIA activates SigG in the forespore and *sigK* encodes the late mother-cell-specific sigma factor SigK. Moreover, SigE-regulated genes contribute to forespore engulfment and sporulation reaches stage III. SigG regulates late-stage sporulation gene expression in the engulfed forespore (Wang *et al.*, 2006). SigG transcribes genes needed for the cortex, and *spoIVB*. SpoIVB, localised in the forespore membrane, plays an essential role in activating SigK in the mother cell (Campo and Rudner, 2007). Hence, SigG is needed to complete stage IV of sporulation and to indirectly activate SigK, the last sigma factor of

the sporulation cascade. Activated SigK is needed for stages V–VII of sporulation. SigK transcribes the *cot* genes, which encode coat proteins, and *spoVD* and *spoVK*, which are responsible for spore maturation (Hilbert and Piggot, 2004). Finally, SigK controls the synthesis of autolysins to ensure the release of the mature spore by mother cell lysis (Lewis, 2000).

### *Differences in the regulation of sporulation in clostridia*

The regulation of sporulation has been studied in detail in the model organism *B. subtilis*. Bacilli and clostridia use the same major sporulation transcription and sigma factors, but class- and species-dependent differences regarding the sporulation programme exist. Most notably, a homologous system to the phosphorelay system initiating sporulation by phosphorylating Spo0A in bacilli could not be detected in clostridia (Paredes *et al.*, 2005; Al-Hinai *et al.*, 2015). Several studies have shown that Spo0A is probably directly phosphorylated by orphan histidine kinases in *C. difficile* (Underwood *et al.*, 2009), *C. acetobutylicum* (Steiner *et al.*, 2011), *C. thermocellum* (Mearls and Lynd, 2014) and *C. botulinum* (Worner *et al.*, 2006). Other important differences in sporulation between bacilli and clostridia concern the temporal pattern of sporulation sigma factor activity, as well as transcriptional and posttranslational regulation of the sporulation sigma factors (Al-Hinai *et al.*, 2015). In *C. botulinum*, the most notable difference compared to the *Bacillus* model in the regulation of sporulation is the dual role of SigK during sporulation: SigK is expressed in early and late stages of sporulation, and analysis of *C. botulinum sigK* mutants suggested that SigK is needed to initiate the sporulation pathway and might be active prior to Spo0A (Kirk *et al.*, 2012; Kirk *et al.*, 2014a). In bacilli, SigK is only active during late-stage sporulation and initiates coat formation (Hilbert and Piggot, 2004). An early role of SigK during sporulation was also described in *C. acetobutylicum* (Al-Hinai *et al.*, 2014) and *C. perfringens* (Harry *et al.*, 2009), but not in *C. difficile* (Pereira *et al.*, 2013). Furthermore, SigG appears to act differently in *C. botulinum*. *C. botulinum sigG* mutants formed engulfed forespores surrounded by a thin coat layer, revealing that SigG is not needed for coat formation (Kirk *et al.*, 2014a). In contrast, *B. subtilis* requires SigG for coat formation (Karmazyn-Campelli *et al.*, 1989), as SigG activates SigK, and SigK initiates coat formation (Hilbert and Piggot, 2004). Therefore, SigG might not be needed for SigK activation in *C. botulinum*. It has to be noted that the studies revealing novel insights into the regulation of sporulation in *C. botulinum* have been conducted in a Group I *C. botulinum* type A strain, and group- or strain-dependent differences cannot be excluded. Altogether, the regulation of sporulation in *C. botulinum* and other clostridia differs substantially from that in bacilli.

### **2.4.5 Role of sporulation in toxin-producing spore formers**

Sporulation and toxin formation are linked in some pathogenic spore formers. The transcriptional regulator Spo0A, the sporulation repressor AbrB and sporulation-specific sigma factors have been shown to play a role in the transcriptional regulation of toxin

synthesis. Sporulation-dependent enterotoxin synthesis is well described in *C. perfringens* (Zhao and Melville, 1998; Huang *et al.*, 2004; Harry *et al.*, 2009). Enterotoxigenic *C. perfringens* causes food poisoning in humans. After ingestion of food contaminated with vegetative cells, the enterotoxin is produced and released by sporulating cells in the gastrointestinal tract and causes the typical symptoms of a gastrointestinal disease, namely diarrhoea and intestinal cramping (Freedman *et al.*, 2016). Enterotoxin synthesis occurs in the mother-cell compartments of sporulating cells and is regulated by the mother-cell-specific sigma factors SigE and SigK, ensuring compartment-specific enterotoxin synthesis and release by mother cell lysis (Harry *et al.*, 2009). A role of Spo0A in regulating toxin gene expression in *C. difficile* has been proposed in the literature, but contradictory data have been published (Rosenbusch *et al.*, 2012; Mackin *et al.*, 2013; Pettit *et al.*, 2014). It appears that the role of Spo0A in toxin gene regulation is strain dependent in *C. difficile* and Spo0A interestingly negatively, positively and not at all influences the production of the toxins, depending on the strain background. In *B. cereus*, cereulide synthesis is directly repressed by the sporulation repressor AbrB, which is under the negative control of Spo0A (Lücking *et al.*, 2009; Ehling-Schulz *et al.*, 2015). In *C. botulinum*, sporulation-dependent BoNT regulation has not been studied.

## 2.5 Importance of *C. botulinum* for food safety

*C. botulinum* spores are widespread in nature. Preventing the contamination of raw food materials with *C. botulinum* is extremely challenging and general hygienic practices can only keep the contamination level low. Therefore, measures eliminating contamination or preventing spore germination have to be taken into account. Spores are sufficiently destroyed by applying the “botulinum cook” (121 °C for 3 min) (Stumbo *et al.*, 1975; Dahlsten *et al.*, 2015). However, insufficient heat treatment and post-processing contamination represent risk factors (Sobel *et al.*, 2004; Peck, 2009). Moreover, not every type of food product is meant to be processed at such a high temperature, and measures preventing the growth of *C. botulinum* must be administered. These measures include the addition of high salt concentration or other preservatives, an acidic pH and cold storage (Sobel *et al.*, 2004; Lindström *et al.*, 2006).

Importantly, Group I and II *C. botulinum* differ in their physiology and therefore possess different risks regarding food safety (Lindström and Korkeala, 2006; Peck, 2009; Carter and Peck, 2015). Group I *C. botulinum* is mesophilic and cannot grow at temperatures lower than 10 °C. Hence, chilled storage prevents Group I *C. botulinum* from growth and toxin production. However, canned or bottled meat and vegetable products are frequently stored at room temperature, and the highly heat-resistant Group I *C. botulinum* spores, which may have survived inadequate heat treatments, might germinate at storage temperatures above 10 °C. Group II *C. botulinum*, known to be psychrotrophic, can grow and produce BoNT at temperatures as low as 3 °C. Temperature abuse in minimally-processed vacuum-packaged foods represents a major risk regarding Group II *C. botulinum*-related foodborne botulism (Lindström *et al.*, 2006; Peck, 2006). Minimally processed foods have become popular during recent years, and so-called refrigerated processed foods of extended durability

(REPFED) have been developed. These foods receive only mild heat treatments and lower concentrations of preservatives. Moreover, vacuum packaging is frequently applied. The mild heat treatments might not be sufficient to kill all spores, and anaerobic storage allows *C. botulinum* spore germination and growth. Therefore, adequate cold storage is very important for the safety of these food products, and it is recommended to store them at temperatures as low as 3 °C or lower (Lindström *et al.*, 2006). Due to the aquatic origin of Group II *C. botulinum* type E, minimally processed fish or seafood products such as vacuum-packaged smoked fish possess a high risk (Hyytiä *et al.*, 1998; Hyytiä *et al.*, 1999).

Other food products with a high risk of Group II-related foodborne botulism are traditionally-prepared uncooked aquatic animals (fish, whale, seal, walrus and beaver) among the indigenous population of northern America (Austin and Leclair, 2011; Fagan *et al.*, 2011; Leclair *et al.*, 2013b). These foods are often placed into sealed bags or nowadays plastic containers and aged for weeks, for example in cool earthen pits, before consumption. Due to the high prevalence of Group II *C. botulinum* type E spores in aquatic environments of the north (Baltic Sea, Canada, Alaska, and Greenland) (Huss, 1980; Hielm *et al.*, 1998; Leclair *et al.*, 2013a; Leclair *et al.*, 2017), marine mammals and fish are frequently contaminated with spores and the special preparation (aging for weeks under anaerobiosis at a low temperature) favours the growth and neurotoxin production of psychrotrophic *C. botulinum* type E strains. For centuries, type E-related foodborne botulism has represented an endemic hazard among indigenous people in the north.

The danger of BoNT production in certain food products has been recognized by the food industry and public health agencies, and appropriate safety measures have been applied to reduce the risk of foodborne botulism outbreaks. However, *C. botulinum* still represents a considerable hazard. Understanding the regulation of BoNT synthesis and the mechanisms contributing to stress tolerance in *C. botulinum*, in particular the mechanisms behind cold stress tolerance, will have an impact on the improvement of food safety measures to further reduce the risk of foodborne botulism outbreaks.

## **2.6 Temperature stress response in *C. botulinum***

Every living organism is affected by ambient temperature and has to react and adapt to changing temperatures to ensure survival and growth under extreme temperatures. The cold- and heat-stress response has been thoroughly studied in many bacteria. The following two chapters will provide an overview of temperature stress tolerance in bacteria with a special focus on *C. botulinum*.

### **2.6.1 Heat stress response**

A high temperature negatively affects the general cellular function of bacteria due to impaired protein stability. Even small increases in temperature lead to misfolding, denaturation and aggregation of proteins, making proper cellular homeostasis impossible (Richter *et al.*, 2010). Additionally, the cell membrane, ribosomes, RNA, and DNA suffer

from heat. Thus, bacteria have to respond to increased temperatures quickly and efficiently to ensure survival. A temperature upshift leads to cellular reorganization at the transcriptional and translational level in order to restore cell functionality (Schumann, 2003; Schumann, 2016). This cellular reorganization is called the heat-shock response. Importantly, the heat-shock response to a large degree mimics other stress responses (Hecker *et al.*, 1996). Therefore, the heat-shock response represents a good model to study the general stress response mechanisms in bacteria. Moreover, stress responses affect virulence in pathogenic bacteria (Anderson *et al.*, 2006; van der Veen *et al.*, 2007). Therefore, understanding how bacteria react to stress conditions contributes to a better understanding of pathogenesis and might enable novel measures to control the conditions favouring pathogenesis.

The heat-shock response is primarily triggered by impaired protein homeostasis and induces the expression of genes encoding so-called heat-shock proteins. In *B. subtilis*, heat-shock proteins are classified according to the transcriptional regulator that controls the expression of the heat-shock protein (Schumann, 2003). Class I and III heat-shock proteins form a cellular protein quality control system and are regulated by HrcA and CtsR, respectively. While class I heat-shock proteins (DnaK and GroEL/ES) act as molecular chaperones facilitating the correct folding of unfolded proteins and refolding of misfolded proteins (Lund, 2001; Hayer-Hartl *et al.*, 2016), class III heat-shock proteins (ATP-dependent Clp proteases) degrade irreversibly damaged proteins (Moliere and Turgay, 2009). The largest group of heat-shock proteins is regulated by the alternative sigma factor SigB and assigned as class II heat-shock proteins (Schumann, 2003). They play a role in the oxidative stress response, transport of compatible solutes, DNA protection, antibiotic resistance and membrane functionality. However, the function of many class II heat-shock proteins is unknown. Importantly, class II heat-shock genes are also expressed in response to many other stresses, such as salt, ethanol, acidity, antibiotics and starvation (Hecker *et al.*, 1996), and have been reported to contribute to virulence in pathogenic bacteria (Hecker *et al.*, 2007). However, a *sigB* homologue is absent in the genomes of *C. botulinum* (Sebahia *et al.*, 2007), and it was suggested that SigK might substitute SigB in regulating stress response-related genes (Dahlsten *et al.*, 2013).

The genome-wide gene expression profile in response to heat has been studied in Group I *C. botulinum* ATCC 3502 (Liang *et al.*, 2013). *C. botulinum* ATCC 3502 was incubated at 37 °C until the mid-exponential growth phase and then heat shocked for 15 min at 45 °C. DNA microarray analysis revealed that 176 genes were differently expressed in response to heat. The heat-shock response led to the overexpression of class I heat-shock genes (*dnaK* and *groELS* operons), but the transcription of class III heat-shock genes was unaffected by heat stress. The induction of class I heat-shock genes after heat shock was also reported by Selby *et al.* (2011), and insertional inactivation of *dnaK* and its transcriptional repressor *hrcA* resulted in reduced stress resistance and decreased maximum growth temperatures compared with the wild type (Selby *et al.*, 2011). Heat shock further caused reduced expression of genes related to translation (aminoacyl-tRNA synthetase genes) and cell division (*ftsZ* and *ftsH*), which suggests growth arrest after heat shock (Liang *et al.*, 2013). Additionally, transcriptomic changes have been observed for genes related to energy metabolism, membrane biogenesis, motility and gene regulation. Sporulation-related genes



and the neurotoxin gene cluster did not show significantly different expression after heat shock. *ha17* and *ha33* were mildly upregulated, suggesting a possible role of the neurotoxin-associated Ha17 and Ha33 proteins in protecting or stabilizing the neurotoxin under high temperatures (Liang *et al.*, 2013). Enhanced Ha33 production after heat shock has also earlier been reported (Shukla and Singh, 2009). In *C. botulinum* strain Hall A batch cultures incubated at 37 °C and 45 °C, neurotoxin cluster genes were not differently expressed (Couesnon *et al.*, 2006). However, reduced BoNT synthesis was observed in *C. botulinum* strain Hall A grown in a fermenter at 45 °C compared to 35 °C (Siegel and Metzger, 1979).

## 2.6.2 Cold stress response

A temperature downshift affects the physiology of bacterial cells. Cold shock decreases membrane fluidity due to the solidification of fatty acids and consequently impairs membrane-associated functions such as uptake and secretion, reduces the flexibility of the secondary structure of RNA and DNA leading to impaired transcription and translation, disturbs protein folding and enzymatic activity, and affects the functionality of ribosomes (Weber and Marahiel, 2003; Phadtare, 2004). This global impact of cold on cellular physiology typically leads to growth arrest, allowing bacterial cells to adapt and regain cellular functionality. During growth arrest, general protein synthesis is extremely reduced and proteins needed to adapt to cold are expressed (Weber and Marahiel, 2003).

In order to restore efficient transcription and translation at a low temperature, destabilization of cold-induced RNA secondary structures is required (Phadtare, 2004). So-called cold-shock proteins and DEAD-box RNA helicases act as RNA chaperones during cold stress. DEAD-box helicases unwind RNA duplexes and contribute to RNA folding (Jones *et al.*, 1996). The important role of DEAD-box helicases in cold tolerance has been shown in various bacteria (Hunger *et al.*, 2006; Markkula *et al.*, 2012; Palonen *et al.*, 2012), including *C. botulinum* (Söderholm *et al.*, 2015). The small highly-conserved cold-shock proteins bind single-stranded RNA and increase RNA flexibility, which improves translation, RNA degradation and the termination of RNA transcription (Ermolenko and Makhatadze, 2002). Group I *C. botulinum* encodes three cold-shock protein genes (*cspA-C*) and CspB was identified as the major cold-shock protein (Söderholm *et al.*, 2011). However, cold-shock proteins are not conserved in *C. botulinum*. Interestingly, psychrotrophic Group II *C. botulinum* type E does not encode any cold-shock protein genes (Söderholm *et al.*, 2013), suggesting that Group II *C. botulinum* might use alternative strategies in destabilizing rigid RNA secondary structures.

Furthermore, cold-stressed bacteria have to restore membrane fluidity by altering the lipid composition of the cell membrane. To regain membrane fluidity at a low temperature, increased amounts of low-melting-point fatty acids, such as unsaturated fatty acids, are needed in the cell membrane. This can be achieved by *de novo* synthesis of unsaturated fatty acids or by desaturation of saturated fatty acids (Suutari and Laakso, 1994; Los and Murata, 1998). Desaturation of membrane fatty acids via a membrane-embedded desaturase under the control of the TCS DesK/DesR has been well described in *B. subtilis* (Aguilar *et al.*, 1999; Aguilar *et al.*, 2001; Mansilla and de Mendoza, 2005). The transmembrane domain

of the membrane-embedded histidine kinase DesK senses the thickness of the membrane (Cybulski *et al.*, 2010; Inda *et al.*, 2014). At the optimum temperature, the “sunken buoy” motif (hydrophilic residues) near the N-terminus of the transmembrane domain is held on the hydrophilic outer surface of the membrane, the water–membrane interface. At a low temperature, the thickness of the membrane is increasing due to more ordered lipid arrangements and the sunken buoy motif sinks deeper into the lipid bilayer, becomes surrounded by hydrophobic parts of the membrane and causes a conformational change in the cytoplasmic domain via straightening of the linker region connecting the transmembrane domain to the cytoplasmic domain (Inda *et al.*, 2014). This conformational change facilitates the switch from phosphatase to kinase activity of the cytoplasmic domain. Subsequently, the cognate response regulator DesR becomes phosphorylated by DesK, and activated DesR induces the transcription of *des* encoding a desaturase. The membrane-embedded  $\Delta 5$ -desaturase introduces double bonds into the fatty acids of the membrane lipids to increase membrane fluidity (Mansilla and de Mendoza, 2005). As fatty acid desaturases are oxygen dependent, no homologous systems have been described in anaerobic bacteria. However, remodelling of the membrane lipid composition with increased levels of unsaturated fatty acids was observed in *C. botulinum* at a low temperature (Russell *et al.*, 1995; Evans *et al.*, 1998). Nevertheless, the system behind temperature-dependent membrane modifications is not known in *C. botulinum*.

Cold-induced TCSs have been identified in Group I *C. botulinum* (Lindström *et al.*, 2012; Derman *et al.*, 2013). The mechanisms activating these TCSs are not known, but their kinases might sense and respond to decreased membrane fluidity in a similar way to DesK. The best studied cold-induced TCS in *C. botulinum* is CBO0366/CBO0365. Expression of the TCS genes *cbo0366* and *cbo0365* in Group I *C. botulinum* ATCC 3502 was induced after cold shock, and insertional inactivation of the TCS genes resulted in cold-sensitive phenotypes (Lindström *et al.*, 2012). Studying the regulon of the response regulator CBO0365 revealed that acetone-butanol-ethanol fermentation, arsenic resistance, motility and phosphate uptake and transport play a role in the cold tolerance of *C. botulinum* (Dahlsten *et al.*, 2014b).

Transcriptomic analysis of *C. botulinum* ATCC 3502 revealed that genes related to the uptake of compatible solutes are induced after cold shock (Dahlsten *et al.*, 2014a). Increased uptake of compatible solutes such as carnitine, betaine and choline may provide cold tolerance via the cryo- and osmoprotective activity of these molecules. Furthermore, the expression of fatty acid biosynthesis genes was altered after cold shock, which might play a role in counteracting cold-induced solidification of membrane lipids. Importantly for a toxin-producing spore former, the expression of *bot* and sporulation-related genes was not affected by cold in this study. However, a separate study demonstrated that the sporulation-specific sigma factor SigK plays an important role during cold and osmotic stress (Dahlsten *et al.*, 2013). Expression of *sigK* was induced upon cold shock and osmotic stress, and *sigK* mutants revealed impaired growth at a low temperature and under hyperosmotic conditions. Interestingly, the late-stage sporulation sigma factor SigK also plays a role in early sporulation in *C. botulinum* ATCC 3502 (Kirk *et al.*, 2012) and might compensate for the general stress sigma factor SigB, which is absent in *C. botulinum* (Sebahia *et al.*, 2007;

Dahlsten *et al.*, 2013). Moreover, SigK might be involved in the decision-making process between the initiation of sporulation and the stress response.

### 3 Aims of the study

The objectives of this thesis study were to investigate the regulation of neurotoxin synthesis and the temperature stress response in *C. botulinum*.

The specific aims were as follows:

1. To examine the role of the master regulator of sporulation Spo0A in neurotoxin synthesis in *C. botulinum* type E.
2. To study the genome-wide transcriptional response to heat in a continuously growing *C. botulinum* ATCC 3502 culture with a special focus on the expression of neurotoxin- and sporulation-related genes.
3. To investigate the involvement of the TCS CLO3403/CLO3404 in the cold tolerance of *C. botulinum* Beluga.

## 4 Materials and methods

### 4.1 Bacterial strains, plasmids, primers and culture (I–III)

The *C. botulinum* strains, *E. coli* strains and plasmids used in studies I–III are listed in Table 2. *C. botulinum* strains were grown in tryptone-peptone-glucose-yeast extract (TPGY) broth (50 g/l tryptone, 5 g/l peptone, 4 g/l glucose, and 20 g/l yeast extract [Difco Becton Dickinson, Sparks, MD, USA] plus 1 g/l sodium thioglycolate [Merck KGaA, Darmstadt, Germany]) or on TPGY agar (1%) under strictly anaerobic conditions in an anaerobic work station (MG1000 anaerobic work station; Don Whitley Scientific Ltd, Shipley, UK) with an atmosphere of 85% N<sub>2</sub>, 10% CO<sub>2</sub> and 5% H<sub>2</sub>. For phenotypic analysis in study II, *C. botulinum* ATCC 3502 cultures were incubated in anaerobic jars under an anaerobic atmosphere created by AnaeroGen sachets (Oxoid Limited, Hampshire, United Kingdom). If not otherwise stated, Group I and Group II strains were grown at 37 °C and 30 °C, respectively. Before use, TPGY broth was deoxygenated by boiling for 15 min and TPGY agar plates were placed for 48 h inside the anaerobic work station. When appropriate, thiamphenicol (15 µg/ml), erythromycin (2.5 µg/ml) and/or cycloserine (250 µg/ml) were added to the media. *E. coli* strains were grown aerobically at 37 °C in LB medium (Difco) or on LB agar supplemented with chloramphenicol (25 µg/ml) and kanamycin (30 µg/ml) for selection. The primers used in this study can be found in the respective original articles (I–III).

**Table 2. Strains and plasmids used in this study.**

Strains and plasmids	Description	Source
<b>Strains</b>		
<i>C. botulinum</i>		
Beluga	Wild-type strain (WT), isolated from beluga flippers	IFR <sup>a</sup>
K3	WT, isolated from rainbow trout surface	DFHEH <sup>b</sup>
11/1-1	WT, isolated from whitefish roe	DFHEH
ATCC 3502	WT, isolated from canned peas?	ATCC <sup>c</sup>
Beluga <i>spo0As</i>	Insertional mutant of <i>spo0A</i> in sense direction at position 519-520	I
Beluga <i>spo0Aa</i>	Insertional mutant of <i>spo0A</i> in antisense direction at position 310-311	I
Beluga-pMTL82151	WT with empty plasmid	I/III
Beluga <i>spo0As</i> -pMTL82151	<i>spo0As</i> with empty plasmid	I
Beluga <i>spo0Aa</i> -pMTL82151	<i>spo0Aa</i> with empty plasmid	I
Beluga-pMTL82151:: <i>spo0A</i>	WT with complementation plasmid	I
Beluga <i>spo0As</i> -pMTL82151:: <i>spo0A</i>	<i>spo0As</i> with complementation plasmid	I
Beluga <i>spo0Aa</i> -pMTL82151:: <i>spo0A</i>	<i>spo0Aa</i> with complementation plasmid	I
K3 <i>spo0As</i>	Insertional mutant of <i>spo0A</i> in sense direction at position 519-520	I
K3 <i>spo0Aa</i>	Insertional mutant of <i>spo0A</i> in antisense direction at position 310-311	I
11/1-1 <i>spo0Aa</i>	Insertional mutant of <i>spo0A</i> in antisense direction at position 310-311	I
Beluga <i>clo3403s</i>	Insertional mutant of <i>clo3403</i> in sense direction at position 630-631	III
Beluga <i>clo3403a</i>	Insertional mutant of <i>clo3403</i> in antisense direction at position 227-228	III
Beluga <i>clo3404s</i>	Insertional mutant of <i>clo3404</i> in sense direction at position 384-385	III
Beluga <i>clo3404a</i>	Insertional mutant of <i>clo3404</i> in antisense direction at position 505-506	III

Beluga <i>clo3403s</i> -pMTL82151	<i>clo3403s</i> with empty plasmid	III
Beluga <i>clo3403a</i> -pMTL82151	<i>clo3403a</i> with empty plasmid	III
Beluga <i>clo3404s</i> -pMTL82151	<i>clo3404s</i> with empty plasmid	III
Beluga <i>clo3404a</i> -pMTL82151	<i>clo3404a</i> with empty plasmid	III
Beluga -pMTL82151:: <i>clo3404-clo3401</i>	WT with complementation plasmid	III
Beluga <i>clo3403s</i> -pMTL82151:: <i>clo3404-clo3401</i>	<i>clo3403s</i> with complementation plasmid	III
Beluga <i>clo3403a</i> -pMTL82151:: <i>clo3404-clo3401</i>	<i>clo3403a</i> with complementation plasmid	III
Beluga <i>clo3404s</i> -pMTL82151:: <i>clo3404-clo3401</i>	<i>clo3404s</i> with complementation plasmid	III
Beluga <i>clo3404a</i> -pMTL82151:: <i>clo3404-clo3401</i>	<i>clo3404a</i> with complementation plasmid	III
<i>E. coli</i>		
<i>E. coli</i> CA434	Conjugation donor	Purdy <i>et al.</i>
NEB 5-alpha Competent <i>E. coli</i>	Cloning strain	New England BioLabs
Rosetta 2(DE3) pLysS	Recombinant expression host	Novagen
Rosetta 2(DE3) pLysS pET28b:: <i>spo0A</i> -DBD	Recombinant expression host harbouring recombinant protein expression vector	I
<b>Plasmids</b>		
pMTL007C-E2	Clo3ron vector for mutagenesis	Heap <i>et al.</i>
pMTL007C-E2:: <i>spo0A</i> -520s	pMTL007C-E2 targeting Beluga <i>spo0A</i> in sense direction	I (DNA2.0)
pMTL007C-E2:: <i>spo0A</i> -311a	pMTL007C-E2 targeting Beluga <i>spo0A</i> in antisense direction	I (DNA2.0)
pMTL007C-E2:: <i>clo3403</i> -631s	pMTL007C-E2 targeting <i>clo3403</i> in sense direction	III (DNA2.0)
pMTL007C-E2:: <i>clo3403</i> -228a	pMTL007C-E2 targeting <i>clo3403</i> in antisense direction	III (DNA2.0)
pMTL007C-E2:: <i>clo3404</i> -385s	pMTL007C-E2 targeting <i>clo3404</i> in sense direction	III (DNA2.0)
pMTL007C-E2:: <i>clo3404</i> -506a	pMTL007C-E2 targeting <i>clo3404</i> in antisense direction	III (DNA2.0)
pMTL82151	Plasmid vector	Heap <i>et al.</i>

pMTL82151:: <i>spo0A</i>	Complementation plasmid containing the Beluga <i>spo0A</i> gene including 422 bp upstream and 143 bp downstream from <i>spo0A</i>	I
pMTL82151:: <i>clo3404-clo3401</i>	Complementation plasmid containing the operon of the TCS CLO3403/CLO3404	III
pET28b	Recombinant protein expression vector	Novagen
pET28b:: <i>spo0A</i> -DBD	pET28b harbouring the C-terminal DNA-binding domain sequence of <i>spo0A</i>	I

<sup>a</sup> Culture Collection of the Institute of Food Research, Norwich, UK. <sup>b</sup> Department of Food Hygiene and Environmental Health, Helsinki, Finland. <sup>c</sup> American Type Culture Collection



## 4.2 Mutagenesis in *C. botulinum* (I, III)

### 4.2.1 Construction of mutants

The ClosTron mutagenesis tool (Heap *et al.*, 2007; Heap *et al.*, 2010) was used to disrupt *spo0A* in *C. botulinum* Beluga, K3, and 11/1-1 and the TCS genes *clo3403* and *clo3404* in *C. botulinum* Beluga. The genes were knocked out by inserting a mobile group II intron from *Lactococcus lactis* (LI.ltrB). For each gene, the intron was inserted in sense and antisense orientations. The intron carries an erythromycin resistance cassette (*erm*), which becomes activated by insertion and facilitates the screening of integrants.

Gene-specific target sites of the introns were designed using the Perutka algorithm at ClosTron.com. Mutagenesis plasmids carrying the retargeted introns (pMTL007C-E2) were ordered from DNA2.0 (Menlo Park, CA, US), transformed into the conjugation donor *E. coli* CA434, and then conjugated into *C. botulinum*. Transconjugants were isolated on TPGY agar containing thiamphenicol (15 µg/ml) and cycloserine (250 µg/ml) and restreaked on TPGY agar containing erythromycin (2.5 µg/ml). Erythromycin-resistant colonies were selected and PCR analysis confirmed correct intron insertion. PCR-confirmed integrants were subjected to Southern blot analysis to verify single intron insertion. Loss of the pMTL007C-E2 plasmids was confirmed by sensitivity to thiamphenicol.

### 4.2.2 Complementation of mutations

The shuttle vector pMTL82151 (Heap *et al.*, 2009) was used to construct complementation plasmids. To complement *spo0A* mutations, a fragment encompassing the coding sequence of *spo0A*, 422 bp upstream of the start codon and 143 bp downstream of the stop codon, was PCR amplified and cloned into pMTL82151. To complement *clo3403* and *clo3404* mutations, a fragment covering the coding sequence of the operon *clo3404-clo3401*, 457 bp upstream and 154 bp downstream of the operon, was PCR amplified and cloned into pMTL82151. The resulting pMTL82151::*spo0A* and pMTL82151::*clo3404-clo3401* plasmids were verified by sequencing and transformed into *E. coli* CA434. The complementation plasmids and empty vector controls were conjugated into the respective wild-type and mutant strains.

## 4.3 Experimental setup to study Spo0A-dependent regulation of BoNT synthesis and sporulation (I)

To study the role of Spo0A in growth, sporulation and neurotoxin production, the *C. botulinum* wild-type strains Beluga, K3 and 11/1-1 and the respective *spo0A* mutant strains

were grown in TPGY broth for 120 h. The growth of each culture was followed by measuring its optical density with a spectrophotometer and by determining the viable cell counts after 10 h, 24 h, 96 h and 120 h. At the same time points, culture samples were withdrawn for neurotoxin analysis by enzyme-linked immunosorbent assay (ELISA). Sporulation was investigated after 120 h of growth by determining the spore concentration (spores/ml) of the cultures using a spore heating assay and by microscopy after staining spores with malachite green. The experiments were performed 3 or 6 times.

#### **4.4 Neurotoxin ELISA (I, II)**

Commercially available BoNT ELISA kits (Tetracore, Rockville, MD, USA) were used to measure BoNT amounts in culture supernatants and lysed cell pellets. The supernatant and cell pellet of each sample (1.5 ml) were separated by centrifugation (10 min, 8200 rpm). Cell pellets were lysed by the addition of 1.5 ml lysis buffer (10 mg/ml lysozyme in 25 mM Tris-HCl [pH 7.5]) and incubation at 37 °C for 1 h (Group I *C. botulinum*) or 2 h (Group II *C. botulinum*) under shaking. Supernatants and crude cell lysates were subjected to neurotoxin ELISA analysis (Bradshaw *et al.*, 2004). ELISAs were performed according to the manufacturer's instructions. The absorbance at 405 nm was measured with a microtiter plate reader (Multiscan Ascent, Thermo Fisher Scientific, Waltham, MA, USA). To calculate toxin concentrations, standard curves of purified BoNT/A complex (kindly provided by Michel R. Popoff, Institute Pasteur, Paris, France) and BoNT/E (Metabio, Madison, WI, USA) were generated. The total amount of BoNT produced in *C. botulinum* cultures was determined by combining the calculated BoNT concentrations of supernatant and cell pellet samples.

#### **4.5 Analysis of sporulation**

##### **4.5.1 Spore heating assays (I, II)**

Culture samples were heat-treated to kill vegetative cells, but not spores. Group I *C. botulinum* culture aliquots were subjected to 80 °C for 15 min and Group II *C. botulinum* culture aliquots were subjected to 60 °C for 20 min. Heat-treated samples were subsequently serially diluted (1:10) into fresh TPGY broth and outgrowth indicated the germination of viable spores. Spore concentrations were calculated using the most-probable-number technique according to the Bacteriological Analytical Manual of the FDA (<http://www.fda.gov/Food/FoodScienceResearch/LaboratoryMethods/ucm109656.htm>). Viable cell counts were obtained similarly by omitting the heat-treatment step.

#### **4.5.2 Spore staining (I)**

Group II *C. botulinum* strains were incubated on TPGY agar plates at their optimum growth temperature of 30 °C for 5 days to guarantee a sufficient time for completion of sporulation. Colonies were then dispensed on glass slides and cells and spores were fixed by flaming. Fixed cells and spores were stained with safranin and malachite green (Schaeffer and Fulton, 1933). Safranin stains vegetative cells red in colour, while malachite green stains mature spores with a developed spore coat green in colour. Stained cells and spores were observed by microscopy.

#### **4.6 Electron microscopy (I, III)**

Spore structures (I) and flagella formation (III) of *C. botulinum* strains were investigated by transmission electron microscopy. *C. botulinum* culture aliquots were sampled and directly mixed (1:1) with 5% glutaraldehyde (Sigma-Aldrich, St. Louis, MO, USA) (III) or cells were harvested by centrifugation (10 min at 3000 x g) and resuspended in a solution containing 2.5% glutaraldehyde and 18.5% formaldehyde (I). In both cases, cell fixation mixtures were incubated for 2 h at room temperature and fixed cells were washed with sterile water.

To investigate the structure of spores (I), fixed cells were embedded in Epon 812 epoxy resin (TAAB Materials, Berks, United Kingdom) and sliced with a diamond knife to obtain ultrathin sections (60 to 80 nm), which were placed on carbon mesh grids and stained with uranyl acetate (0.5%) and Pb-citrate (1%) for 30 min. Embedding, slicing and staining of the cells and spores was performed by the staff of the Electron Microscopy Unit of the Institute of Biotechnology, University of Helsinki.

To investigate flagella formation (III), 3.5 µl of the fixed cell suspensions were dropped on carbon-coated grids (Electron Microscopy Sciences, Hatfield, PA, USA) and incubated for 2 min. Thereafter, the liquid was removed and 3.5 µl phosphotungstic acid (1%) was placed on the grid to stain the cells for 15 s.

In both studies (I and III), the prepared grids were analysed using a Tecnai 12 transmission electron microscope (Philips Electron Optics, Eindhoven, Holland) in the Electron Microscopy Unit of the Institute of Biotechnology, University of Helsinki.

#### **4.7 Motility assay (III)**

To investigate TCS CLO3403/CLO3404-dependent motility phenotypes at different temperatures, single colonies of the respective *C. botulinum* strains grown on TPGY agar plates were stabbed with a sterile inoculation loop into 0.3% TPGY agar and incubated at 30 or 12 °C under anaerobic conditions. Diffusion of *C. botulinum* into the agar indicated a motile phenotype. Growth and motility were observed daily over 4 days.

## 4.8 Characterization of growth phenotypes at low and optimum growth temperatures (III)

*C. botulinum* cultures were diluted (1:100) into fresh TPGY broth and 350 µl of each dilution was pipetted into 4 wells of a microtiter plate (technical replicates). The microtiter plate was placed in a Bioscreen C Microbiology Reader (Oy Growth Curves AB, Helsinki, Finland) inside an anaerobic work station. The plates were incubated at 12 °C for 10 days or at 30 °C for 2 days and the optical densities of each well were automatically measured. To calculate the maximum growth rate (OD<sub>600</sub> value per hour) of each culture, the respective growth curves were fitted to the Baranyi and Roberts model (Baranyi and Roberts, 1994) using the DMFit Microsoft Excel add-in program (Institute of Food Research, Norwich, UK). The experiment was performed in triplicate.

## 4.9 Protein–DNA interaction (I)

### 4.9.1 Protein expression and purification

The C-terminal DNA-binding domain of Spo0A (Spo0A DBD) was linked with a histidine tag at its N-terminus. The DNA sequence corresponding to the Spo0A DBD was PCR-amplified and cloned into the expression vector pET28b to label the recombinant protein with 6 histidines. The correct sequence was confirmed by sequencing the resulting pET28b-*spo0A* DBD. Thereafter, the plasmid was transformed into *E. coli* Rosetta 2(DE3) pLysS cells (Novagen, Madison, WI, USA). *E. coli* Rosetta 2(DE3) pLysS harbouring pET28b-*spo0A* DBD was incubated at 37 °C in 100 ml LB until reaching an optical density of 0.8, and protein expression was induced with 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) for 5 h. Cells were harvested by centrifugation (15 min, 10,000 x g, 4 °C), dissolved in lysis/binding buffer (500 mM NaCl, 20 mM imidazole, 20 mM Tris-HCl, pH 7.9) and lysed by sonication. After removing cell debris by centrifugation (15 min, 10,000 x g, 4 °C) and filtering, the supernatant was mixed with 1 ml Novagen His Bind affinity resin and loaded onto a gravity flow chromatography column (Thermo Fisher Scientific). Bound his-tagged Spo0A DBD was washed with 9 ml lysis/binding buffer and 15 ml washing buffer (500 mM NaCl, 60 mM imidazole, 20 mM Tris-HCl, pH 7.9), and purified Spo0A DBD was subsequently eluted with 4 ml elution buffer (500 mM NaCl, 500 mM imidazole, 20 mM Tris-HCl, pH 7.9). The purity of the eluted protein was confirmed by SDS-PAGE. Finally, the recombinant Spo0A DBD suspension was filled into Novagen D-tube dialyzers (molecular mass cutoff 6 to 8 kDa) and dialysis into storage buffer (50 mM HEPES [pH 7.8], 100 mM NaCl, 5 mM MgCl<sub>2</sub>, 1 mM EDTA, 10% glycerol) occurred overnight at 4 °C. The protein concentration was determined with a Qubit 2.0 fluorometer (Invitrogen).

#### 4.9.2 Electrophoretic mobility shift assay (EMSA)

EMSAs were performed to study the binding capability of recombinant Spo0A DBD to the *botE* promoter (*PbotE*). *PbotE* probes were constructed by PCR amplification with 5'-end biotin-labelled forward primer *PbotE-F* biotin and the reverse primer *PbotE-R* (Table S2 in study I). *PbotE* probes harbouring mutated versions of the putative Spo0A-binding box were constructed with ordered DNA fragments (Eurofins Genomics, Ebersberg, Germany) containing the desired nucleotide changes. A probe encompassing the promoter of the *abrB* gene (*PabrB*) was similarly constructed and served as a positive control. As negative controls, a probe encompassing a part of the coding sequence within *botE* and a probe encompassing the putative promoter region of the spore germination endopeptidase encoding *gpr* (*Pgpr*) were constructed. To my knowledge, *gpr* has never been reported to be under the direct control of Spo0A, and *Pgpr* represents a suitable negative control promoter.

Biotin-labelled probe was mixed with 0 to 5  $\mu\text{M}$  recombinant Spo0A DBD in binding buffer (20 mM HEPES [pH 7.8], 12 mM KCl, 5 mM  $\text{MgCl}_2$ , 1 mM EDTA, 1 mM DTT, 0.3 mg of BSA/ml, 10% glycerol) containing 1  $\mu\text{g}$  poly(dI-dC) (Thermo Fisher Scientific). Binding reactions were incubated at 25  $^\circ\text{C}$  for 1 h and then resolved on 5% native polyacrylamide gels running in 0.5 x Tris-borate-EDTA (TBE) buffer at 4  $^\circ\text{C}$  for 3 h at 80 V. Labelled DNA probes were blotted on positively charged nylon membranes (Roche Applied Science, Penzberg, Germany) and cross-linked. For visualization, a chemiluminescent nucleic acid detection module kit (Thermo Fisher Scientific) was used according to the manufacturer's instructions.

#### 4.10 Heat-shock experiment (II)

To study genome-wide heat stress response, *C. botulinum* ATCC 3502 was grown in continuous culture in a 5-l Braun Biostat B fermentor (B. Braun, Melsungen, Germany) in 2 l of TPGY broth buffered with 6.25 g/l  $\text{NaH}_2\text{PO}_4$  and 5.45 g/l  $\text{KH}_2\text{PO}_4$  (VWR International, Leuven, Belgium). The culture was incubated at 39  $^\circ\text{C}$ , stirred at 200 rpm and supplemented with  $\text{N}_2$  to secure anaerobiosis. To verify anaerobiosis, resazurin sodium salt (1 mg/ml; Sigma-Aldrich) was used as an indicator. The culture pH was constantly kept at 6.8 by the automatic addition of 3 M KOH (Sigma-Aldrich) and foam formation was suppressed with Antifoam A (20 mg/ml; Sigma-Aldrich). The optical density of the culture was automatically measured and converted to absorption units (AU). After reaching an optical density of 1.5 AU, feeding was conducted at a dilution rate of 0.035  $\text{h}^{-1}$ . About 24 h after feeding was started, a constant optical density of 1.6 to 1.7 AU was reached, which indicated steady-state growth. The control sample (5 ml) was taken and the temperature was increased to 45  $^\circ\text{C}$ . After 8 min, the culture reached 45  $^\circ\text{C}$  and the first heat-shock sample was taken. Further samples were taken 10 min, 1 h, 18 h and 42 h after heat shock and the last sample was withdrawn after the culture reached steady-state continuous growth at 45  $^\circ\text{C}$  with a stable optical density of 0.7 to 0.8 AU. The culture samples (5 ml) were immediately mixed with 2 ml stop solution and incubated for 30 min on ice. The culture

supernatant was removed after centrifugation at  $5000 \times g$  at  $4\text{ }^{\circ}\text{C}$  for 5 min, and the cell pellet was stored at  $-70\text{ }^{\circ}\text{C}$ . The experiment was performed twice (biological replicates), and at each sampling time point, two samples were taken (technical replicates).

#### **4.11 Cold-shock experiment (III)**

To study the role of the TCS CLO3403/CLO3404 in the cold-shock response, *C. botulinum* Beluga was grown in TPGY broth at  $30\text{ }^{\circ}\text{C}$  until the culture reached an optical density of 1.0 to 1.2. Subsequently, the culture was divided into two cultures and one of them was placed in an ice-water bath. The temperature of the culture was measured with a sterile thermometer. After the temperature of the culture reached  $12\text{ }^{\circ}\text{C}$ , the culture was further incubated at  $12\text{ }^{\circ}\text{C}$  for 24 h. The non-shocked control culture was incubated at  $30\text{ }^{\circ}\text{C}$  for the same period of time. To study cold-related expression of the TCS genes *clo3403* and *clo3404*, samples for transcriptional analysis were taken from both cultures immediately before and 1 min, 30 min, 1 h, 2 h, 5 h and 24 h after cold shock. Samples were mixed (6:1) with an ethanol (99%)/phenol (95%) solution (9:1) and incubated for 30 min at  $4\text{ }^{\circ}\text{C}$ . After centrifugation (5 min, 8000 rpm,  $4\text{ }^{\circ}\text{C}$ ), the supernatant was removed and pelleted cells were stored at  $-70\text{ }^{\circ}\text{C}$ . The experiment was performed in triplicate.

#### **4.12 Transcriptional analysis**

##### **4.12.1 RNA isolation and cDNA synthesis (II, III)**

Frozen cell pellets were lysed for 30 min (II) or 2 h (III) at  $37\text{ }^{\circ}\text{C}$  in lysis buffer (25 mg/ml lysozyme and 250 IU/ml mutanolysin in Tris-EDTA [pH 8.0]). Total RNA was extracted with RNeasy Mini (III) or Midi (II) Kits (Qiagen, Hilden, Germany) according to the manufacturer's instructions. During RNA isolation, genomic DNA was removed by DNase treatment (RNase free DNase set; Qiagen). A second DNase treatment (DNA free; Ambion, Life Technologies Corporation, Carlsbad, CA, USA) was performed after RNA elution. Concentrations of the RNA samples were measured with a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific) and RNA quality was assessed by electrophoresis with an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA).

Purified RNA (800 ng) was reverse transcribed into cDNA by using the DyNAmo cDNA Synthesis Kit (Thermo Scientific) according to the manufacturer's instructions. Before cDNA synthesis, RNA secondary structures were reduced by denaturation for 5 min at  $65\text{ }^{\circ}\text{C}$ . Reverse transcription (RT) was performed in duplicate for each RNA sample (technical replicates) and DNA contamination was controlled by RT reactions without adding reverse transcriptase. cDNA samples were stored at  $-20\text{ }^{\circ}\text{C}$ .

#### 4.12.2 Real-time quantitative reverse transcription PCR (RT-qPCR) (II, III)

RT-qPCR was performed in duplicate for each cDNA sample using the Maxima SYBR Green qPCR Kit. The PCR reactions included 1 × Maxima SYBR Green qPCR master mix, 0.3 μM of each primer, 4 μl of diluted cDNA and water. The PCR run included one cycle at 95 °C for 1 min (II) or 10 min (III), 40 cycles at 95 °C for 15 s (III) or 10 s (II) and 60 °C for 60 s (III) or 20 s (II), and a final cycle at 60 °C for 1 min. No-template controls were performed with each run to rule out contamination of the reagents. The specificity of the PCR products was confirmed after the run by melting curve analysis. To produce the melting curves, the samples were heated from 60 °C to 98 °C at 0.5 °C intervals for 10 s. The efficiency of the PCR reactions was determined for each primer pair by constructing standard curves with serially diluted cDNA pools. To normalize gene expression, the 16S *rrn* expression values were used (Kirk *et al.*, 2014b). Relative gene expression was calculated according the Pfaffl method (Pfaffl, 2001).

#### 4.12.3 DNA microarray analysis (II)

The effect of heat stress on the gene expression profile of *C. botulinum* ATCC 3502 was studied with ATCC 3502 custom-designed *in situ*-synthesized DNA microarrays (8 × 15 K, Agilent Technologies). The DNA microarrays covered 3641 chromosomal (out of 3648) and 19 plasmid-borne CDSs of the *C. botulinum* ATCC 3502 genome (Sebahia *et al.*, 2007; Dahlsten *et al.*, 2014b).

Two micrograms of total RNA from each sample were reverse transcribed into cDNA and directly labelled with the fluorescent dyes Cy3 or Cy5. To construct labelled cDNA, the RNA was mixed with 5 μg of random primers, 40 U of RNaseOUT Recombinant Ribonuclease Inhibitor, 6 μl of 5 × first-strand buffer, 3 μl of 100 mM DTT, 400 U of SuperScript III Reverse Transcriptase (all Invitrogen, Life Technologies Ltd, Paisley, UK), 0.6 μl of dNTP mix (25 mM each of dATP, dGTP and dTTP and 10 mM dCTP [Promega Corporation, Madison, WI, USA]), and 2 nmol of Cy3-dCTP or Cy5-dCTP (GE Healthcare, Buckinghamshire, UK) in a final volume of 30 μl. The reaction mixture was incubated for 3 h at 46 °C. Subsequently, 1.5 μl of 20 mM EDTA and 15 μl of 0.1 M NaOH were added and the mixture was incubated for 15 min at 70 °C. After RNA hydrolysis, the samples were neutralized by adding 15 μl of 0.1 M HCl. Purification of labelled cDNA was performed with DNA purification columns (QIAquick PCR Purification Kit, Qiagen). Finally, labelled cDNA was eluted into 44 μl of elution buffer (Qiagen).

As a next step, 300 ng of Cy3-labelled cDNA and 300 ng of Cy5-labelled cDNA were mixed. After adding 2.3 μg of salmon sperm DNA (Invitrogen), the samples were denatured by incubation for 2 min at 95 °C and then cooled on ice. Thereafter, 10 × blocking agent (Agilent Technologies) and 2 × RPMI hybridization buffer (Agilent Technologies) was added. A volume of 50 μl of the cDNA mix was loaded on the DNA microarray slide and hybridized for 16 h at 65 °C. Washing was performed according to the manufacturer's instructions. Each cDNA sample of the culture grown at 45 °C was hybridized against the

control sample. To obtain technical replicates, the dyes were also swapped. Additionally, dye bias was controlled on each slide with differently labelled control samples.

Scanning of the DNA microarray slides was performed at 532 nm and 635 nm in an Axon GenePix Autoloader 4200 AL scanner (Axon Instruments, Westburg, Leusden, The Netherlands) using a 5  $\mu$ M resolution. Images were processed with Gene Pix Pro 6.0 software (Axon Instruments), and the R limma package (Smyth and Speed, 2003; Smyth, 2005) was used for data analysis. Foreground and local background intensities of each spot were determined by the mean and median pixel values of the spot. After subtracting the local background signal from the foreground signal by following the “normexp” method with an offset value of 50 (Ritchie *et al.*, 2007), the signal intensities calculated for the Cy3 and Cy5 channels were converted into a logarithmic ( $\log_2$ ) scale and normalized by the Loess method (Smyth and Speed, 2003).

To determine genes differently expressed in samples taken after temperature upshift to 45 °C compared to the control sample taken before temperature upshift, statistical analysis using the R limma package was conducted (Smyth, 2005). *P*-values for each probe were obtained by using a moderated *t*-test with empirical Bayes variance shrinkage (“eBayes” function) and converted into false discovery rate (FDR) values using the Benjamini–Hochberg adjustment. Each CDS was represented by the probe with the median unmodified *P*-value for the expression difference. CDS having an expression difference of  $\log_2$ -ratio  $\geq 1$  or  $\leq -1$  and  $\text{FDR} \leq 0.05$  were considered to be differently expressed at 39 °C and 45 °C. The differently expressed CDSs were clustered according to the expression pattern over time. The k-means clustering method with Euclidean distance was applied using the MultiExperimentViewer of the TM4 Microarray Software Suite (Saeed *et al.*, 2003).



## 5 Results

### 5.1 The sporulation transcription factor Spo0A directly activates neurotoxin synthesis in *C. botulinum* type E (I)

#### 5.1.1 Spo0A is needed for efficient neurotoxin synthesis and sporulation in *C. botulinum* type E

Insertional inactivation of *spo0A* in *C. botulinum* Beluga resulted in impaired sporulation and highly reduced BoNT/E synthesis compared to the wild type (Fig. 2 and Fig. 5 in study I). The *C. botulinum* Beluga *spo0As* and *spo0Aa* mutants produced less than 10% of the wild-type BoNT/E level within 120 h of growth, suggesting that Spo0A is needed for efficient neurotoxin synthesis. This was confirmed by successful complementation of *spo0A*: introducing a plasmid harbouring *spo0A* under its natural promoter (pMTL82151::*spo0A*) produced significantly increased BoNT/E levels compared to vector control strains.

Sporulation was only restored in the complemented *spo0Aa* mutant ( $6 \times 10^4$  spores/ml), whereas the complemented *spo0As* mutant did not form spores (Fig. 2 in study I). Transmission electron microscopy revealed that the complemented *spo0As* mutant initiated sporulation and formed engulfed forespores (Fig. 3 in study I). However, the spore cortex and coat were not built and no mature spores were produced. It thus seems that the role of Spo0A in the initiation of sporulation was successfully restored by introducing pMTL82151::*spo0A*, but completion of sporulation was blocked at stage III after forespore engulfment. Interestingly, the complemented *spo0As* mutant with restored BoNT production and blocked sporulation released a much smaller proportion of BoNT to the extracellular environment than the wild-type strain, suggesting that factors downstream of Spo0A in the sporulation cascade or completion of sporulation are responsible for efficient BoNT release.

Phenotypic characterization of *spo0A* mutants of two more *C. botulinum* type E strains (K3 and 11/1-1) showed that a functional *spo0A* is also needed in those strains for efficient BoNT production and sporulation (Fig. S1 in study I). Therefore, the role of Spo0A as a positive regulator of BoNT synthesis seems to be conserved in *C. botulinum* type E.

#### 5.1.2 The C-terminal DNA-binding domain of Spo0A (Spo0A DBD) binds specifically to the putative Spo0A-binding box (CTTCGAA) within the *botE* promoter (*PbotE*)

The electrophoretic mobility shift assay (EMSA) was performed to test whether Spo0A activates BoNT expression by directly binding to the putative Spo0A-binding box (CTTCGAA) within *PbotE*. Binding of the recombinant Spo0A DNA-binding domain

(Spo0A DBD) was tested to a probe encompassing wild-type *PbotE*, to three *PbotE* probes with mutated versions of the putative Spo0A-binding box, and to a positive control probe encompassing the putative promoter region of *abrB* (*PabrB*), which contains a perfect match with the conserved Spo0A-binding box (TGTCGAA) and has been shown to bind Spo0A DBD in multiple organisms (Strauch *et al.*, 1990; Fujita *et al.*, 2005; Rosenbusch *et al.*, 2012).

Recombinant Spo0A DBD caused a shift in migration of the *PabrB* probe, confirming that *PabrB* is a target of Spo0A in *C. botulinum* Beluga and a suitable positive control probe (Fig. 6 in study I). Remarkably, the wild-type *PbotE* probe harbouring the putative Spo0A-binding box (CTTCGAA), which contains two mismatches with the conserved Spo0A-binding box sequence (TGTCGAA), was also shifted by Spo0A DBD. To test whether Spo0A DBD specifically binds to the putative Spo0A-binding box, EMSA with *PbotE* probes containing mutated versions of the Spo0A-binding box was performed. *PbotE* probes lacking the putative Spo0A-binding box sequence and *PbotE* probes containing seven random nucleotides (GAGAATT) instead of the putative Spo0A-binding box sequence were not bound by Spo0A DBD, suggesting that Spo0A DBD binds specifically to the putative Spo0A-binding box within *PbotE*. Shifted protein–DNA complexes were not observed with negative control probes. Altogether, Spo0A appears to bind specifically to the putative Spo0A-binding box within *PbotE* in *C. botulinum* type E Beluga.

### **5.1.3 The putative Spo0A-binding box (CTTCGAA) is conserved in *botE* promoters (*PbotE*)**

The sequence and location of the putative Spo0A-binding box within *PbotE* appears to be conserved among strains producing BoNT/E. In *C. botulinum* type E strains Beluga, Alaska, K3 and 11/1-1 and *C. butyricum* BL5262 producing BoNT/E, the same putative Spo0A-binding box (CTTCGAA) is located 131 bp upstream of the start codon of the *botE* operon, which suggests a conserved role of Spo0A in activating BoNT/E synthesis. This conserved role of Spo0A in BoNT/E regulation is supported by decreased BoNT/E synthesis in *C. botulinum* K3 and 11/1-1 *spo0A* mutant cultures compared to the respective wild-type strain cultures (Fig. S1 in I).

## 5.2 Heat has a negative effect on neurotoxin synthesis and sporulation in *C. botulinum* ATCC 3502 (II)

### 5.2.1 Expression of neurotoxin cluster genes and sporulation-related genes is suppressed during the heat-shock response in continuously growing *C. botulinum* ATCC 3502

To investigate the effect of high temperature on the gene expression profile of *C. botulinum*, the *C. botulinum* strain ATCC 3502 was grown in continuous culture until reaching a stable optical density of 1.6 to 1.7 AU and subsequently heat-shocked by increasing the growth temperature from 39 to 45 °C. After the heat shock, the optical density declined and stabilized around 80 h thereafter, indicating adaptation to the elevated temperature. Samples for DNA microarray analysis were withdrawn immediately before heat shock, right after the culture reached 45 °C, 10 min, 1 h, 18 h and 42 h thereafter, and after the culture had adapted to heat and grew continuously at a stable optical density. Differences in gene expression between samples taken before and after the heat shock were expected to be exclusively due to heat shock, since growth phase-dependent factors, such as acid stress or starvation, were probably excluded in the continuous culture experiment.

Expression levels of selected, significantly heat-affected genes can be found in Table S1 (II) and expression levels of all studied genes are shown in Table S2 (II).

Expression of BoNT cluster genes, which are localized on the chromosome in two adjacent operons (*ntnh-botA* and *ha33-ha17-ha70*), was significantly downregulated after heat treatment compared to pre-heat-shock: by 1.4- to 2.7-fold after 10 min, 5.2- to 7.5-fold after 1 h, 3.0- to 4.6-fold during heat adaptation and 5.1- to 6.8-fold after re-adapting to heat. Surprisingly, the expression of *botR*, encoding the neurotoxin gene cluster associated alternative sigma factor BotR, was not suppressed during heat. However, heat stress appears to have a negative effect on BoNT production in *C. botulinum* ATCC 3502.

Sporulation allows bacteria to survive unfavourable environmental conditions, including high temperature. However, a sudden increase in the growth temperature led to significant transcriptional suppression of 56 out of 88 sporulation-associated genes, which started in most cases 1 h after the heat shock and continued until the end of the experiment. These repressed sporulation genes included the four sporulation sigma factor genes *sigF*, *sigE*, *sigG* and *sigK*: *sigK* was already suppressed from 1 h after heat shock onwards, whereas the expression of *sigF*, *sigE* and *sigG* was mainly repressed during and after heat adaption. The expression of *spo0A*, which encodes the master regulator of sporulation, was downregulated immediately after the heat shock and in the heat-adapted culture. Altogether, sporulation appears to be avoided during heat stress.

In addition to the repression of neurotoxin genes and genes related to sporulation, heat shock negatively affected the transcription of genes related to transcription and translation (e.g. genes coding for RNA polymerases, 30S and 50S ribosomal proteins, and aminoacyl tRNA synthetases), which explains the observed growth arrest after heat exposure. It also induced the expression of class I and III heat-shock genes, which indicates that our experimental setup successfully induced a heat-shock response in *C. botulinum*. Moreover,

the heat shock affected genes related to carbon and protein metabolism. Expression analysis of genes related to the acetone-butanol-ethanol pathway revealed that after heat stress, during adaption to heat, and also after adaption to heat, *C. botulinum* ATCC 3502 favours ethanol as a fermentation end product instead of butanol and butyrate. Several changes in the fermentation pathways of certain amino acids were also observed during heat adaptation. Additionally, our DNA microarray analysis revealed that more than half of the 88 chemotaxis- and flagellar-related genes were induced at least at one time point after heat shock. These genes included genes for chemotaxis, structural flagellar genes, genes for flagellar assembly and biosynthesis, and the flagellar operon-specific sigma factor *sigD*. Hence, increased motility appears to play a role during the heat-shock response.

To validate the temperature-induced changes in gene expression determined by DNA microarray analysis, we performed relative gene expression analysis by RT-qPCR of selected genes at two time points. The results obtained by microarray analysis were confirmed for the selected genes by RT-qPCR (Fig. 4 in study II), suggesting that the whole-genome transcriptional analysis was conducted in an appropriate way.

### **5.2.2 Neurotoxin synthesis and sporulation are significantly reduced at a high temperature (45 °C) in *C. botulinum* ATCC 3502 batch cultures**

The influence of high temperature treatment (45 °C) on BoNT production and sporulation in *C. botulinum* ATCC 3502 batch cultures was studied at the phenotypic level using neurotoxin ELISAs and sporulation heating assays, respectively. Measured BoNT concentrations were significantly lower in *C. botulinum* ATCC 3502 batch cultures incubated at 45 °C than in cultures incubated at 39 °C, confirming the negative effect of high temperature treatment on BoNT production at the protein level (Fig. 5A in study II). Spores could not be detected in cultures incubated at 45 °C, whereas  $1.2 \times 10^6$  spores/ml were detected in cultures grown at 39 °C (Fig. 5B in study II). Hence, BoNT production and sporulation were both repressed by heat in *C. botulinum* ATCC 3502 batch cultures. These results are in line with the reduced expression of the neurotoxin gene cluster operons and sporulation-related genes in continuously growing heat-shocked *C. botulinum* ATCC 3502 cultures and suggest a possible regulatory link between BoNT production and sporulation in *C. botulinum* ATCC 3502.

### **5.3 The TCS CLO3403/CLO3404 of *C. botulinum* Beluga is important for the cold-shock response and growth at a low temperature (III)**

#### **5.3.1 Transcription of the TCS genes *clo3403* and *clo3404* is induced after cold shock**

A cold shock (temperature downshift from 30 °C to 12 °C) induced the expression of *clo3403* and *clo3404* by 1.5- to 3.4-fold during the first 5 h after cold shock relative to the expression before cold shock (Fig. 2 in study III). In the non-shocked culture, *clo3403* and *clo3404* expression increased from mid-exponential growth (time point  $t_0$ ) to late-exponential growth (time point 1 h). However, after entry into stationary growth, transcription of the TCS genes declined strongly at time point 2 h and onwards. In contrast, in the cold-shocked culture, the expression of *clo3403* and *clo3404* remained induced at time points 2 h and 5 h, suggesting that the TCS CLO3403/CLO3404 plays a role in adaptation to cold stress. At 5 h, an over 100-fold induction of *clo3403* and *clo3404* expression was observed in the cold-shocked cultures relative to the expression in the non-shocked control cultures. Prolonged expression of the TCS CLO3403/CLO3404 after cold shock suggests that the TCS and/or members of the regulon of the TCS are needed for optimal growth during cold exposure.

#### **5.3.2 The *clo3403* and *clo3404* mutants show impaired growth at a low temperature (12 °C), but not at the optimal temperature (30 °C)**

Growth of the *clo3403* and *clo3404* mutants was impaired at a low temperature (12 °C) compared with wild-type growth (Fig. 4 in study III). TCS mutants cultured at 12 °C displayed an extended lag phase and/or lower maximum optical densities compared to the wild-type strain. Additionally, maximum growth rates of all TCS mutants were significantly ( $P < 0.01$ ) lower compared to the wild type (Table 3 in study III). At 30 °C, growth curves and maximum growth rates of the TCS mutants were very similar to those of the wild type. Complementation of the mutations confirmed the important role of the TCS CLO3403/CLO3404 in efficient growth at a low temperature (Fig. 5 and Table 3 in study III).

#### **5.3.3 The cold-sensitive *clo3403s* mutant is not motile and does not form flagella**

*C. botulinum* Beluga and the *clo3404* response regulator mutants were fully motile at 12 °C and 30 °C, but the *clo3403s* histidine kinase mutant revealed a non-motile phenotype at both temperatures (Fig. 6 in study III) due to abolished flagellar synthesis (Fig. 7 in study III). Motility and flagellar synthesis were restored in the *clo3403s* histidine kinase mutant by

introducing the complementation plasmid pMTL82151::*clo3404-clo3401* (Fig. 6 and Fig. 7 in study III). Interestingly, the *clo3403s* mutant exhibited the most impaired growth at a low temperature, with the lowest maximum growth rate among all mutants (Table 3 in study III). Therefore, the cold-sensitive phenotype of *clo3403s* might be at least partly caused by impaired flagellar synthesis.

## 6 Discussion

### 6.1 Sporulation and neurotoxin synthesis are co-regulated via Spo0A in *C. botulinum* type E (I)

The characterization of *C. botulinum* type E *spo0A* mutants revealed that Spo0A is required for sporulation and efficient BoNT synthesis. EMSA demonstrated that Spo0A directly activates BoNT/E synthesis by binding to a putative Spo0A-binding box (CTTCGAA) that resides 131 bp upstream of the *botE* operon in *C. botulinum* Beluga. The same Spo0A-binding box is located at the same chromosomal position in other BoNT/E-producing clostridia, suggesting a conserved role of Spo0A in neurotoxin regulation in *C. botulinum* type E. Interestingly, the putative Spo0A-binding box sequence (CTTCGAA) is almost palindromic and resides more or less in the middle of the non-coding region between the *botE* operon and the *orfX* operon, which are transcribed in opposite directions (see Fig. 6B in study I). Hence, Spo0A might bind to both DNA strands in the Spo0A-binding box region and could activate the transcription of both operons, the *botE* operon and the *orfX* operon. Spo0A is the first neurotoxin regulator reported in *C. botulinum* type E and represents a regulatory link between sporulation and BoNT synthesis. In *C. botulinum* type A, sporulation and BoNT synthesis were suppressed at a high temperature (Fig. 5 in study II) and both traits were reported to be influenced by quorum sensing (Cooksley *et al.*, 2010). Therefore, common regulation of sporulation and BoNT synthesis might be conserved in *C. botulinum*.

In addition to BoNT production, BoNT release might also be connected to sporulation. In the complemented Beluga *spo0As* mutant, sporulation was blocked at stage III after forespore engulfment, and restored BoNT levels were not released to the extracellular environment. In contrast, the Beluga wild type and the complemented *spo0Aa* mutant formed heat-resistant spores and released a major amount of the produced neurotoxin. The unexpected phenotype (blocked sporulation and impaired BoNT release) of *spo0As* pMTL82151::*spo0A* was possibly caused by an undesired polar effect of the strong *erm* resistance cassette promoter within the inserted intron (Heap *et al.*, 2007; Heap *et al.*, 2010), which may have silenced the expression of the upstream gene *spoIVB*. Silencing of *spoIVB*, a gene that is essential for the activation of late sporulation sigma factor SigK in *B. subtilis* (Campo and Rudner, 2007), might have blocked the sporulation cascade before distinctive cortex and coat formation and subsequently mother cell lysis due to the suppression of SigK-dependent autolysin synthesis (Lewis, 2000). This might explain the high intracellular amounts of BoNT detected in *spo0As* pMTL82151::*spo0A* and suggests that BoNT is produced in sporulating cells and released by mother cell lysis. However, activation of BoNT synthesis could be Spo0A-level dependent. High threshold levels of phosphorylated Spo0A are probably needed to initiate sporulation (Fujita *et al.*, 2005), while low amounts of phosphorylated Spo0A might be sufficient for the activation of *bot* transcription. Therefore, we cannot exclude the possibility that, in addition to sporulating cells, vegetative cells also produce and release BoNT. Determination of the cell type (vegetative cells and/or

sporulating cells) producing BoNT and identification and characterization of the mechanism(s) releasing BoNT remain to be studied in more detail.

Co-regulation of sporulation and BoNT synthesis via Spo0A might have a positive effect on the survival and transmission of *C. botulinum* in nature. Sporulation and BoNT synthesis in anaerobic decomposing materials, such as carcasses, might initiate animal botulism outbreaks (Espelund and Klaveness, 2014; Rossetto *et al.*, 2014; Montecucco and Rasotto, 2015). When vertebrates ingest spores and BoNT from contaminated feed or prey, the action of the neurotoxin will kill the animals and turn them into anaerobic fermenters, allowing spore germination and growth. After vegetative growth and the consumption of nutrients, *C. botulinum* produces spores and BoNT, which might again be ingested by animals. Natural botulism, especially among birds, can spread rapidly and affect many individuals. Spo0A-dependent co-regulation of sporulation and BoNT synthesis might contribute to the efficiency of botulism outbreaks in nature by ensuring the formation of spores surrounded with BoNT.

In such a scenario, BoNT represents the key for spore germination after transmission, and it would actually make sense for *C. botulinum* to produce spores covered with BoNT. The outermost layer of *C. botulinum* spores is the exosporium (Hodgkiss and Ordal, 1966; Hawirko *et al.*, 1972; Masuda *et al.*, 1980), which can be seen in Fig. 6B of study I. Exosporia have a high protein content, are occasionally irregularly shaped, and are loosely attached to the spore (Stewart, 2015). BoNT as part of the exosporium would provide several advantages for the organism: (i) BoNT is ultimately linked to the spore through physical contact, increasing the probability of common transmission, (ii) BoNT is protected from harsh conditions in the environment, and (iii) BoNT can be released, for example by decomposition of the exosporium, without decreasing spore resistance. However, whether BoNT is part of the exosporium or any other component of the spore remains to be studied.

Sequence analysis of *PbotE* revealed that there are several transcriptional binding sites of DNA regulators directly downstream of the putative Spo0A-binding box (Fig. S2 in study I). We found putative binding sites for the transition-state regulator CodY (Belitsky and Sonenshein, 2008), the sporulation repressor AbrB (Chumsakul *et al.*, 2011), the sporulation-specific sigma factor SigK (Haldenwang, 1995) and the alternative sigma factor UviA (Dover *et al.*, 2014). All of these DNA-binding regulators have been reported to play a role in the toxigenesis of pathogenic spore-formers (Dupuy *et al.*, 2005; Dineen *et al.*, 2007; Harry *et al.*, 2009; Lücking *et al.*, 2009; Frenzel *et al.*, 2012; Dover *et al.*, 2014; Zhang *et al.*, 2014), and Spo0A-binding boxes (having maximal 1 mismatch with the conserved box) are present in the promoter regions of the genes encoding the aforementioned regulators, suggesting that they might be under the direct control of Spo0A. Hence, Spo0A appears to play a master regulatory role in BoNT/E regulation. The described model of transcriptional regulation of BoNT/E synthesis (for details, see Fig. S2 in study I) shows that up to three sporulation-related regulators (Spo0A, SigK and AbrB) might control BoNT/E synthesis. This suggests a strong linkage between the two traits.



## 6.2 Heat stress in *C. botulinum* ATCC 3502 cultures suppresses neurotoxin and sporulation gene expression and induces motility-related gene expression (II)

Heat shock in continuously growing *C. botulinum* ATCC 3502 caused a growth arrest in combination with the repression of genes related to transcription and translation, induced the expression of the well-known class I and III heat-shock genes to restore protein homeostasis, and affected the expression of genes related to metabolism. Importantly for a spore-forming pathogen, a high temperature appeared to have a negative effect on sporulation and neurotoxin synthesis. Heat shock in continuously growing *C. botulinum* ATCC 3502 reduced the expression of sporulation-related genes, including the four sporulation sigma factor genes (*sigF*, *sigE*, *sigG*, and *sigK*) and the gene encoding the master regulator of sporulation (*spo0A*). Additionally, both operons of the neurotoxin gene cluster (*ntnh-botA* and *ha33-ha17-ha70*) were significantly downregulated after heat shock. Abolished sporulation and significantly reduced BoNT synthesis was further demonstrated in *C. botulinum* ATCC 3502 batch cultures incubated at 45 °C compared with cultures incubated at 39 °C. Reduced BoNT synthesis at a high temperature has also previously been reported for a *C. botulinum* Hall A strain grown in a fermentor (Siegel and Metzger, 1979).

Sporulation is most likely avoided under heat stress, because sporulation is highly energy- and time-consuming and might not be reasonable in cells struggling with protein and DNA damage (Ireton and Grossman, 1994). Spores harbouring DNA with severe mutations would not serve the purpose of preserving intact genetic material for the next generations. The reduced expression of sporulation-related genes might also have caused reduced neurotoxin gene expression, as sporulation and neurotoxin synthesis appear to be co-regulated in *C. botulinum*. Furthermore, the expression of known *bot* transcription activators (*botR*, *codY* and the TCS regulator genes *cbol041* and *cbol968*) (Marvaud *et al.*, 1998b; Connan *et al.*, 2012; Zhang *et al.*, 2014) was unaffected or upregulated after heat shock. Heat-affected expression of the only known negative regulator, the TCS *cbol0787/cbol0786* (Zhang *et al.*, 2013b), could not be determined due to a too low hybridization signal strength. Therefore, it cannot be excluded and even appears likely that BoNT synthesis in *C. botulinum* ATCC 3502 is, in addition to the known BoNT regulators, under control of other regulators, such as sporulation-related transcriptional regulators.

In *C. botulinum* type E, BoNT synthesis is directly activated by Spo0A. In *C. botulinum* ATCC 3502, the expression of both operons of the neurotoxin gene cluster was downregulated from 10 min after heat shock onwards, and the expression of *spo0A* was downregulated immediately after heat shock and in the heat-adapted culture, suggesting that Spo0A might also induce BoNT synthesis in *C. botulinum* ATCC 3502. Additionally, *sigF*, *sigE*, *sigG* and *sigK* encoding sporulation sigma factors were downregulated during and after heat adaption. In particular, SigK, which activates enterotoxin synthesis in *C. perfringens* (Harry *et al.*, 2009), represents an interesting candidate for *bot* regulation. Moreover, expression of the sporulation repressor *abrB* (*cbol0063*), which represses toxin synthesis in *B. cereus* (Lücking *et al.*, 2009) and possibly in *C. botulinum* type E (AbrB binding site exists immediately downstream of the start codon of the *botE* operon; see Fig. S2 in study I), was induced 2.3- and 2.0-fold in the last two time points. However, the factors

contributing to sporulation-dependent BoNT synthesis in *C. botulinum* ATCC 3502 remain to be identified.

A high temperature activated during and after heat adaptation the expression of more than half of the 84 chemotaxis- and flagella-related genes in *C. botulinum* ATCC 3502, including *sigD*, which encodes the chemotaxis- and motility-specific sigma factor (Marquez *et al.*, 1990; Marquez-Magana and Chamberlin, 1994). The activation of motility and repression of sporulation suggest opposite regulation of the two traits during heat stress in *C. botulinum*. Opposite regulation of sporulation and motility has been described in other bacteria (Tomas *et al.*, 2003; Lulko *et al.*, 2007) and was linked to reduced competition for SigD in conditions with reduced expression of sporulation sigma factor genes (Lulko *et al.*, 2007). When facing high temperatures, increased motility might allow *C. botulinum* to search for less stressful environments (with temperatures closer to their optimal growth temperature) for growth and potentially BoNT synthesis and sporulation. Interestingly, Spo0A inhibits the transcription of genes related to flagellar biosynthesis and chemotaxis in *B. subtilis* (Molle *et al.*, 2003a; Fujita *et al.*, 2005) and *C. difficile* (Pettit *et al.*, 2014). Expression of *spo0A* was reduced immediately after heat shock and in heat-adapted *C. botulinum* ATCC 3502 culture, and expression of the orphan histidine kinase *cbo1120*, which possibly phosphorylates Spo0A (Worner *et al.*, 2006), was downregulated 1.7- and 3.0-fold at time points 1 h and 18 h, respectively. Hence, the reduced expression and phosphorylation of Spo0A during heat stress might increase motility and decrease sporulation and potentially BoNT synthesis.

### **6.3 The TCS CLO3403/CLO3404 of *C. botulinum* Beluga is important for cold tolerance (III)**

In contrast to a high temperature, a low temperature does not reduce the expression of the BoNT- and sporulation-related genes (Chen *et al.*, 2008; Dahlsten *et al.*, 2014a). The ability to grow and efficiently produce BoNT at a low temperature makes psychrotrophic Group II *C. botulinum* type E an especially serious health threat in minimally-processed chilled foods. As cold does not reduce the expression of the neurotoxin gene, understanding of the mechanisms facilitating growth at low temperatures is of particular interest for the food industry to develop measures preventing the growth and BoNT synthesis of *C. botulinum* during cold storage. The cold-shock response and mechanisms facilitating cold tolerance have been studied intensively in mesophilic Group I *C. botulinum* type A (Söderholm *et al.*, 2011; Dahlsten *et al.*, 2013; Derman *et al.*, 2013; Dahlsten *et al.*, 2014a; Dahlsten *et al.*, 2014b; Söderholm *et al.*, 2015), but are unknown in psychrotrophic Group II strains, which do not encode cold-shock protein genes (Söderholm *et al.*, 2013). We identified a TCS contributing to cold tolerance in psychrotrophic Group II *C. botulinum* type E.

Expression of the TCS genes *clo3403* and *clo3404* was induced for 5 h after cold shock relative to their expression before cold shock. In the non-shocked control culture, relative expression levels of *clo3403* and *clo3404* were already downregulated at time points 2 h and 5 h. The prolonged induction of *clo3403* and *clo3404* expression after cold shock suggests that this TCS might play a role in the adaptation of *C. botulinum* Beluga to growth

at low temperatures. Phenotypic analysis of *clo3403* and *clo3404* mutants at a low (12 °C) and the optimal (30 °C) temperature confirmed the important role of the TCS in growth at low temperatures. TCSs important for the cold-shock response and growth at low temperatures have been reported in various bacteria (Ullrich *et al.*, 1995; Aguilar *et al.*, 2001; Chan *et al.*, 2008; Palonen *et al.*, 2011), including Group I *C. botulinum* ATCC 3502 (Lindström *et al.*, 2012; Derman *et al.*, 2013). Investigation of the sensing mechanism of the histidine kinase CLO3403 and identification of the genes under the control of the response regulator CLO3404 will enable a better understanding of cold tolerance in Group II *C. botulinum* type E strains.

The operon encoding the TCS genes *clo3403* and *clo3404* also contains a gene for a substrate-binding protein of an ABC transporter (*clo3401*) and a gene encoding a conserved hypothetical protein of unknown function with a predicted transmembrane helix (*clo3402*). The molecules transported by the ABC transporter substrate-binding protein CLO3401 are unknown. However, it is plausible that CLO3402 and CLO3401 are part of the cold tolerance mechanism mediated by CLO3403 and CLO3404. In *C. botulinum* ATCC 3502, a cold shock induced the expression of the *cbo0558A-cbo0560* operon encoding a DNA regulator and components of a putative ABC transporter system (Dahlsten *et al.*, 2014a). Inactivation of *cbo0558A* resulted in impaired growth at low temperatures. Moreover, the phosphate ABC transporter substrate-binding protein PhoT was shown to be important for growth at low temperatures (Dahlsten *et al.*, 2014b). Functional analysis of CLO3402 and CLO3401 may reveal further insights into the mechanisms contributing to cold tolerance in *C. botulinum* type E.

## 7 Conclusions

1. The sporulation master regulator Spo0A directly activates BoNT synthesis in *C. botulinum* type E. Phenotypic characterization of *spo0A* mutants demonstrated that Spo0A is needed for BoNT synthesis, and *in vitro* binding assays revealed that Spo0A activates the transcription of the *botE* operon by binding to a putative Spo0A-binding box (CTTCGAA) within the *botE* promoter. With Spo0A, a neurotoxin gene regulator was identified for the first time in *C. botulinum* type E and a genetic link between sporulation and BoNT synthesis was found.
2. Sporulation and BoNT synthesis are suppressed at high temperatures. The expression of sporulation-related genes and the BoNT gene *botA* was downregulated after heat shock in a continuously growing *C. botulinum* ATCC 3502 culture. Moreover, in *C. botulinum* ATCC 3502 batch cultures incubated at 45 °C, sporulation was abolished and BoNT synthesis significantly reduced compared with cultures incubated at 39 °C. Downregulation of sporulation and BoNT synthesis at a high temperature suggests that the two traits might be co-regulated in *C. botulinum* ATCC 3502. Furthermore, the expression of motility- and chemotaxis-related genes was upregulated during and after heat adaptation in continuously growing *C. botulinum* ATCC 3502 cultures, suggesting that motility represents a survival strategy during heat stress.
3. The TCS CLO3403/CLO3404 is important for cold tolerance in *C. botulinum* Beluga. We demonstrated the induction and prolonged expression of *clo3403* and *clo3404* during the cold-shock response and impaired growth of *clo3403* and *clo3404* mutants at a low temperature compared to the Beluga wild type. The necessity of the TCS CLO3403/CLO3404 for optimal growth at a low temperature was confirmed by successful complementation of the mutations.

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