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# **Growth temperature variation and heat stress response of *Clostridium botulinum***

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ACADEMIC DISSERTATION

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To my family

## ABSTRACT

*Clostridium botulinum*, the causative agent of botulism in humans and animals, is frequently exposed to stressful environments during its growth in food or colonization of a host body. The wide genetic diversity of the strains of this foodborne pathogen has been thoroughly studied using different molecular biological methods; however, it is still largely unknown how this diversity reflects in the ability of different *C. botulinum* strains to tolerate environmental stresses. In contrast to cold tolerance, which has been the focus of intensive research in recent years, the molecular mechanisms *C. botulinum* utilizes in response to heat shock and during adaptation to high temperature stress are poorly understood. The aims of this study were to investigate the strain variation of Group I and II *C. botulinum* with regard to growth at low, high, and optimal temperature; the roles of *hrcA*, the negative regulator of Class I heat shock genes (HSG) and *dnaK*, a molecular chaperone coding Class I HSG, in the response of the Group I *C. botulinum* strain ATCC 3502 to heat and other environmental stresses; and the molecular mechanisms this strain employs in response to acute and prolonged heat stress.

The maximum and minimum growth temperatures of 23 Group I and 24 Group II *C. botulinum* strains were studied. Further, maximum growth rates of the Group I strains at 20, 37, and 42 °C and of the Group II strains at 10, 30, 37, and 42 °C were determined. Within their groups, the *C. botulinum* strains showed significant variation in growth-limiting temperatures and their capability to grow at extreme temperature, especially at high temperature. Largest strain variation was found for Group I within type B and for Group II within type E strains, which further showed more mesophilic growth tendencies than the other Group II strains. However, the genetic background of the selected *C. botulinum* strains reflected only weakly in their growth characteristics. Group I strains showed larger physiological variation despite being genetically more closely related than Group II. A number of strains of both groups showed faster growth at temperatures above than at their commonly assumed optimal growth temperatures of 30 °C for Group II and 37 °C for Group I strains. In addition, they possessed higher maximum growth temperatures than the average of the studied strains. These strains can be expected to have higher than assumed optimal growth temperatures and pronounced high temperature stress tolerance. Good correlation was detected between maximum growth temperatures and growth rates at high temperature, although not for all strains. Therefore direct prediction from one studied growth trait to the other was impossible. These findings need to be taken into account when estimating the safety of food products with regard to *C. botulinum* by risk assessment and challenge studies.

The role of Class I HSGs in *C. botulinum* Group I strain ATCC 3502 was studied by quantitative real-time reverse transcription PCR and insertional inactivation of the Class I HSGs *hrcA* and *dnaK*. During exponential and transitional growth, Class I HSGs were constantly expressed followed by down-regulation in the stationary phase. Exposure of mid-exponentially growing culture to heat shock led to strong, transient Class I HSG up-

regulation. Inactivation of *hrcA* resulted in over-expression of all Class I HSGs, which confirmed its role as negative regulator of Class I HSGs in *C. botulinum*. Both inactivation mutants showed impaired high temperature tolerance as indicated by reduced growth rates at 45 °C, a reduced maximum growth temperature, and increased log-reduction after exposure to lethal temperature. The growth of the *dnaK* mutant was more strongly affected than that of the *hrcA* mutant, emphasizing the importance of the molecular chaperone DnaK for *C. botulinum*. Reduced growth rates were evident for both mutants under optimal conditions and heat stress, but also under low pH, and high saline concentration. This suggests a probable role for Class I HSG in cross protection of *C. botulinum* against other environmental stresses.

*C. botulinum* ATCC 3502 was grown in continuous culture and exposed to heat shock followed by prolonged high temperature stress at 45 °C. Changes in the global gene expression pattern induced by heat stress were investigated using DNA microarray hybridization. Class I and III HSGs, as well as members of the SOS regulon, were employed in response to acute heat stress. High temperature led to suppression of the botulinum neurotoxin coding *botA* and the associated non-toxic protein-coding genes. During adaptation and in the heat-adapted culture, motility- and chemotaxis-related genes were found to be up-regulated, whereas sporulation related genes were suppressed. Thus, increase in motility appeared to be the long-term high-temperature stress-response mechanism preferred to sporulation. Prophage genes, including regulatory genes, were activated by high temperature and might therefore contribute to the high temperature tolerance of *C. botulinum* strain ATCC 3502. Further, remodeling of parts of the protein metabolism and changes in carbohydrate metabolism were observed.

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## LIST OF ORIGINAL PUBLICATIONS

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

- I. Hinderink K., Lindström M. & Korkeala H. 2009. Group I *Clostridium botulinum* strains show significant variation in growth at low and high temperature. *Journal of Food Protection* 72: 375-383.
- II. Derman Y., Lindström M., Selby K. & Korkeala H. 2011. Growth of Group II *Clostridium botulinum* strains at extreme temperature. *Journal of Food Protection* 74: 1797-1804.
- III. Selby K., Lindström M., Somervuo P., Heap JT., Minton NP. & Korkeala H. 2011. Important role of Class I heat shock genes *hrcA* and *dnaK* in the heat shock response and the response to pH and NaCl stress of Group I *Clostridium botulinum* strain ATCC 3502. *Applied and Environmental Microbiology* 77: 2823-2830.
- IV. Selby K., Mascher G., Somervuo P., Lindström M. & Korkeala H. 2016. Heat shock and prolonged heat stress attenuate neurotoxin gene expression and sporulation machinery gene expression in Group I *Clostridium botulinum* strain ATCC 3502. Submitted for publication.

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## ABBREVIATIONS

ACh	Acetylcholine
AFLP	Amplified fragment length polymorphism
ANTP	Associated non-toxic proteins
BoNT	Botulinum neurotoxin
cDNA	Complementary DNA
CDS	Coding sequences
DNA	Deoxyribonucleic acid
EDTA	Tris-ethylenediaminetetraacetic acid
FDR	False discovery rate
GTP	Guanosine-triphosphate
HA	Hemagglutinin
HC	Heavy chain
HSG	Heat shock gene
IPTG	Isopropyl- $\beta$ -D-thiogalactopyranoside
LC	Light chain
max GR	Maximum growth rate
NTC	Neurotoxin gene cluster
NTNH	Non-toxic non-HA
OD <sub>600</sub>	Optical density at the wavelength of 600 nm
ODU	Optical density units
PCR	Polymerase chain reaction
qPCR	Quantitative real-time PCR
REPFED	Refrigerated processed foods of extended durability
RNA	Ribonucleic acid
rRNA	Ribosomal ribonucleic acid
RT	Reverse transcription
RT-qPCR	Quantitative real-time reverse transcription PCR
Sig	RNA polymerase sigma factor
SNAP-25	Synaptosomal associated protein of 25 kDa
SNARE	Soluble N-ethylmaleimide-sensitive factor attachment protein receptors
TCS	Two-component signal transduction system
T <sub>max</sub>	Maximum growth temperature
T <sub>min</sub>	Minimum growth temperature
T <sub>inc</sub>	Incubation temperature
TPGY	Tryptone-peptone-glucose-yeast extract
VAMP	Vesicle associated membrane protein

# 1. INTRODUCTION

*Clostridium* is a genus of Gram-positive, rod-shaped, obligate anaerobe, endospore-forming bacteria which is widespread in nature. The genus includes a number of highly pathogenic, toxin-producing species (Hatheway, 1990), the most important of which are: *Clostridium perfringens*, the cause of histotoxic and enteric diseases; *C. difficile*, the cause of the emerging antibiotic-associated pseudomembranous colitis; *C. tetani*, the cause of tetanus; and *C. botulinum*, the cause of the different forms of botulism (Hatheway, 1990). This study focuses on the *C. botulinum*.

The botulinum neurotoxin (BoNT) forming *C. botulinum* has first been epidemiologically linked to a foodborne intoxication in 1895/1896 when van Ermengem, a Belgian professor of bacteriology at the University of Ghent, was able to isolate a Gram-positive, anaerobic, spore-forming organism from smoked ham as well as deceased participants of a funeral at which this ham was served (Ermengem, 1897; Erbguth, 2004; Erbguth, 2008). The affected people had died after developing symptoms of a disease commonly called “sausage poisoning”. He called the bacterium *Bacillus botulinus* (from the Latin word *botulus*, meaning “sausage”) (Ermengem, 1897; Torrens, 1998); later it was renamed to *C. botulinum*. The “sausage poisoning”, a paralytic, potentially-lethal disease, had emerged strongly in the late 18<sup>th</sup> and early 19<sup>th</sup> century in the German Kingdom of Württemberg and was soon connected to smoked blood-sausages that had been prepared under poor hygienic conditions and were often under-cooked. The first two detailed clinical descriptions were published in 1817 by Steinbuch and by Kerner (Erbguth & Naumann, 1999; Erbguth, 2009). The latter further conducted pioneering scientific experiments and was able to induce symptoms of botulism in different animals and himself using extracts of spoiled sausages (Kerner, 1822). This led him to the conclusion that the substance in question was a toxin. He further speculated a potential therapeutic use of this poison in the treatment of neurological diseases (Kerner, 1822). Although initially thought to be related to animal products only, botulism was observed after the consumption of canned vegetables and the first environmental strains of *C. botulinum* were isolated (Burke, 1919a; Erbguth, 2009). In addition to the above-described foodborne form of botulism, which is intoxication with BoNT, infections with *C. botulinum* can lead to *in situ* toxin production, namely infant, intestinal and wound botulism (Hatheway, 1990; Sobel, 2005).

Even though food contaminated with BoNT has been linked to botulism for approximately 200 years, foodborne botulism still poses a substantial hazard to human health in modern times (Lindström & Korkeala, 2006; Lindström *et al.*, 2006; Peck *et al.*, 2011; Carter & Peck, 2015). Due to limited possibilities to treat clinical botulism (Sobel, 2005), and the high toxicity of BoNT, the only way to prevent the disease is to ensure the absence of BoNT in food. Many efforts have been made to control and to prevent growth of *C. botulinum* in food. In the early days, rising public awareness and recommendations of outbreak control were the first attempts to reduce the number of botulism cases (Thom,

1922; Hall, 1943). Later, the introduction of the botulinum cook in the canning industry to reduce spore numbers led to a significant decrease in outbreaks caused by commercial products (Stumbo *et al.*, 1975; Peck, 2009; Setlow & Johnson, 2013; Dahlsten *et al.*, 2015). Today, challenge studies, predictive modelling, and modern risk assessment, as well as hurdle technology of mildly-processed convenient foods are some of the methods employed to ensure food safety with regard to botulism (Juneja & Marks, 1999; Lindström *et al.*, 2006; Peck, 2006; Peck *et al.*, 2008; Anderson *et al.*, 2011; Ihekweba *et al.*, 2016; Ihekweba *et al.*, 2016). Nevertheless, outbreaks of botulism are still frequently reported, and a number of them are caused by commercial products (Sobel *et al.*, 2004; Lindström & Korkeala, 2006; Centers for Disease Control and Prevention (CDC), 2011; Daminelli *et al.*, 2011; Jalava *et al.*, 2011; Carter & Peck, 2015)

Differences between *C. botulinum* strains were first observed when antitoxins produced against one strain failed to protect animals against another, as the strains produced serologically distinct toxins. This led to the distinction of different *C. botulinum* serotypes (Burke, 1919b). Further, heterogeneity in their physiology, especially in cell metabolism and nutrient requirements, was identified and the strains were later separated into different groups (Holdeman & Brooks, 1970; Hatheway, 1990). Even though strain variation within these groups (growth temperature and toxin formation) has been reported (Jensen *et al.*, 1987) and the wide genetic diversity of *C. botulinum* has been recognized (Hielm *et al.*, 1998a; Hyytiä *et al.*, 1999; Keto-Timonen *et al.*, 2005; Nevas *et al.*, 2005; Hill *et al.*, 2015; Williamson *et al.*, 2016), little is known about strain variation within groups of *C. botulinum* with regard to growth temperatures (Stringer *et al.*, 2013). Chill temperature is one of the most important hurdles to prevent growth of *C. botulinum* especially in minimally-processed foods, whereas high temperature stress is frequently experienced by foodborne pathogens during food preparation and preservation. Therefore variation in the temperature stress tolerance of different *C. botulinum* strains can have a substantial impact on food safety and its assessment. The impact of cold stress on *C. botulinum* and the molecular mechanisms the bacterium employs have been recently studied thoroughly (Söderholm *et al.*, 2011; Lindström *et al.*, 2012; Dahlsten *et al.*, 2014; Dahlsten *et al.*, 2014; Mascher *et al.*, 2014; Söderholm *et al.*, 2015). However, little is known about the molecular basis of heat stress response in *C. botulinum* to date (Shukla & Singh, 1999; Shukla & Singh, 2009; Liang *et al.*, 2013). Deeper knowledge on strain variation of *C. botulinum* and better understanding of its stress-response mechanisms are therefore needed to enhance food safety with regard to this challenging pathogen.

## 2. REVIEW OF THE LITERATURE

### 2.1. *Clostridium botulinum* and botulism

#### 2.1.1. *Clostridium botulinum*

**Organism.** *Clostridium botulinum* belongs to the genus *Clostridium* of the phylum Firmicutes. Clostridia are a group of Gram-positive, rod-shaped, low G+C deoxyribonucleic acid (DNA)-containing, motile, obligate anaerobe bacteria, which can form heat-resistant endospores (Cato *et al.*, 1986). The bacterium is of a ubiquitous nature, spores of *C. botulinum* are commonly found in soil and aquatic environments in many parts of the world (Hauschild, 1989). The denominating feature of all strains of the species *C. botulinum* is their ability to form botulinum neurotoxin (BoNT) during vegetative growth, the causative agent of a rare, but potentially-lethal neuroparalytic disease called botulism, affecting humans and animals (Prévot, 1953; Hatheway, 1990). Strains of *C. botulinum* are being assigned to the serotypes A-G due to the serological properties of the produced BoNT and separated due to their physiological and metabolic characteristics into Group I-IV (Hatheway, 1990).

*C. botulinum* strains typically carry one neurotoxin-coding gene and therefore express one BoNT serotype. However, bivalent strains carrying two active neurotoxin genes (Ab, Af, Ba, and Bf) (Peck, 2009; Hill & Smith, 2013), and one strain harboring even three (A2f4f5) (Dover *et al.*, 2013; Kalb *et al.*, 2014), have been described. These multiple neurotoxin-gene-carrying strains predominantly form toxin of one serotype, the major toxin, indicated by an upper case letter, and only small amounts of active minor toxin, indicated by a lower case letter. The second neurotoxin gene can also remain unexpressed, thus be silent, which is indicated with brackets in such cases.

The obvious diversity and heterogeneity of the species warranted the differentiation of *C. botulinum* strains into groups, based on their physiological behavior (Holdeman & Brooks, 1970). To date, BoNT-producing clostridia are separated into six groups of which Groups I to IV consist of *C. botulinum* strains, whereas BoNT producing *Clostridium butyricum* and *Clostridium baratii* strains form their own groups (Hatheway, 1990) (Table 1). These phenotypical groups also reflect the phylogenetic background of *C. botulinum* strains, which has been underlined by studies using molecular typing, DNA sequencing (16S ribosomal ribonucleic acid), and DNA hybridization methods (Suen *et al.*, 1988; Hutson *et al.*, 1993; Collins *et al.*, 1994; Keto-Timonen *et al.*, 2005; Hill *et al.*, 2007). The availability of genomic data for a growing number of *C. botulinum* strains, as well as analyses of strains by comparative genomic hybridization using DNA microarrays, have provided deeper insight into the *C. botulinum* phylogeny (Hill *et al.*, 2007; Carter *et al.*, 2009; Lindström *et al.*, 2009; Carter & Peck, 2015; Hill *et al.*, 2015; Williamson *et al.*,

2016). These studies have shown relatively close relatedness within, and high diversity between, the groups. This further fueled the discussion about *C. botulinum* nomenclature and the question of whether the groups should in fact be assigned to different species, as already proposed when Suen et al. suggested Group IV *C. botulinum* as the species *C. argentinense* (Suen et al., 1988; Collins & East, 1998; Peck, 2009).

The groups of BoNT-producing clostridial strains most commonly related to human botulism are Group I (proteolytic *C. botulinum*) and II (non-proteolytic *C. botulinum*), but cases caused by strains of Group V (*C. butyricum*) and VI (*C. baratii*) have also been reported. Group III strains have commonly been associated to botulism in animals and are therefore of economic importance when effecting production animals, whereas Group IV (*C. argentinense*) strains have never been shown to naturally induce disease.

**Physiology.** The characteristic physiological properties of the different groups of BoNT producing clostridial strains are listed in Table 1. Given that Group I and II *C. botulinum* strains are accountable for the majority of human botulism cases, the characteristics of these two groups will be described here in more detail.

All Group I and Group II *C. botulinum* strains are able to ferment glucose, liquefy gelatin, produce lipase, and degrade chitin (Carter & Peck, 2015).

Group I *C. botulinum* is proteolytic, mesophilic, and forms spores extremely resistant to high temperatures and other environmental stresses like radiation, high pressure, and desiccation (Lindström & Korkeala, 2006; Peck, 2009; Johnson, 2013; Setlow & Johnson, 2013). The group consists of strains producing BoNT serotypes A, B, and/or F; all bi- or trivalent strains discovered to date belong to this group (Peck, 2009; Dover et al., 2013; Hill & Smith, 2013). Proteolytic strains of *C. botulinum* have been linked to foodborne, wound, and also to intestinal/infant botulism. Cases of foodborne botulism caused by proteolytic strains most commonly involve home-canned meat and vegetables or commercial products intended to be stored at ambient temperature that had been exposed to process failure (Lindström & Korkeala, 2006; Peck, 2009).

The main characteristic of Group I *C. botulinum* is its distinctive proteolytic activity, which differentiates it from Group II. Group I *C. botulinum* can utilize native protein sources for growth; it is able to digest casein, meat, and coagulated egg white, in addition to other substrates (Holdeman & Brooks, 1970; Hatheway, 1990). Indeed, sequencing the genome of the strain ATCC 3502 revealed the presence of several coding sequences (CDSs) for secreted proteases and a large number of transporters related to peptide and amino acid uptake (Sebahia et al., 2007). Differences in the ability of the various *C. botulinum* strains to ferment amino acids has been used to develop a polymerase chain reaction (PCR) based assay to distinguish Group I from Group II (Dahlsten et al., 2008). The organism is further able to metabolize selected amino acids in a coupled oxidation–reduction reaction, the Stickland reaction (Stickland, 1934; Clifton, 1940; Sebahia et al., 2007). The main end products of its catabolism are acetate, butyrate, ammonia, carbon dioxide, hydrogen, and lactic acids (Clifton, 1940). The ability to ferment carbohydrates is limited and varies between strains. However, all strains are reported to ferment glucose

**Table 1.** Characteristics of botulinum neurotoxin (BoNT) forming clostridia

	Group I <i>C. botulinum</i> (proteolytic)	Group II <i>C. botulinum</i> (non-proteolytic)	Group III <i>C. botulinum</i>	Group IV <i>C. botulinum</i> ( <i>C. argentinense</i> )	<i>C. butyricum</i>	<i>C. baratii</i>
BoNT produced	A,B,F	B,E,F	C, D	G	E	F
Proteolysis of						
Casein	+ <sup>b</sup>	-	+/-	+	-	-
Gelatin	+	+	+	+	-	-
Fermentation of						
Glucose	+	+	+	-	+	+
Sucrose	-	+	-	-	+	+
Lipase production	+	+	+	-	-	-
Optimum growth temperature	37 °C (35-40 °C) <sup>c</sup>	25-30 °C (18-30 °C)	40 °C (35-40 °C)	37 °C (35-40 °C)	30-37 °C (30-45 °C)	30-37 °C (30-40 °C)
Maximum growth temperature	48 °C	45 °C	ND	45 °C	~40 °C	
Minimum growth temperature	10-12 °C	2.5-3 °C	15 °C	12 °C	10 °C	20 °C
Inhibitory NaCl concentration in water phase	10%	5%	3%	6.5% (>3%)	~5%	5%
Minimum growth permitting pH	4.6	5	5.1	ND	~3.6	4.8
Spore heat resistance <sup>d</sup>	D <sub>121</sub> °C = 0.21 min	D <sub>82.2</sub> °C = 2.4 min	D <sub>104</sub> °C = 0.9 min	D <sub>104</sub> °C = 1.1 min	ND	D <sub>100</sub> °C < 0.1 min

Source: Table adapted from references (Hatheway, 1990; Johnson, 2000; Lindström & Korceala, 2006; Peck, 2009; Johnson, 2013; Carter & Peck, 2015)  
<sup>a</sup> ND, no data available; <sup>b</sup> +, all strains positive; -, all strains negative; +/- some of the strains positive, some negative; <sup>c</sup> values in brackets indicate variation between different sources; <sup>d</sup> D, decimal reduction time: time to ten-fold reduction in viable spores at given temperature at pH 7 in phosphate buffer



with ethanol and carbon dioxide as metabolic end products, whereas sucrose and mannose cannot be utilized (Clifton, 1940; Holdeman & Brooks, 1970; Carter & Peck, 2015). Even though carbohydrates can stimulate growth and toxin production of Group I *C. botulinum*, they are not essential for this bacterium (Siegel & Metzger, 1979; Siegel & Metzger, 1980; Whitmer & Johnson, 1988).

Group I *C. botulinum* is considered to be a mesophile, with a commonly-assumed optimum growth temperature of 37 °C (Hatheway, 1993; Peck, 2009). The temperature range enabling growth and toxin formation of these strains is commonly reviewed to be between 10-12 °C and 48 °C (Ohye & Scott, 1953; Lynt *et al.*, 1982; Hatheway, 1990; Johnson, 2000; Johnson, 2000; Peck *et al.*, 2011; Johnson, 2013; Carter & Peck, 2015). However, although extensive studies evaluating a large number of Group I strains have never been performed, temperature limits for the growth of *C. botulinum* appear to vary between different strains (Bonventre & Kempe, 1959b; Jensen *et al.*, 1987; Hauschild, 1989; Hauschild, 1989; Johnson, 2000). The strains tolerate relatively low water activity. Provided otherwise optimal conditions, 10% NaCl in the water phase leading to a water activity of 0.94 is considered to be growth limiting (Lynt *et al.*, 1982; Hauschild, 1989). An environmental pH of 4.6 is assumed to prevent growth of Group I *C. botulinum* (Peck, 2009), although growth and toxin formation in a defined medium at a pH as low as 4.3 have been reported (Nobumasa, 1982).

In contrast to Group I, Group II *C. botulinum* is non-proteolytic, considered to be psychrotrophic, and forms spores substantially less resistant to heat than Group I spores (Lindström *et al.*, 2006; Peck, 2009). Strains of Group II isolated until today produce a single BoNT of serotype B, F, or E and have been associated almost exclusively to foodborne botulism. Non-proteolytic *C. botulinum* is often related to outbreaks involving minimally processed, vacuum-packed, chilled, or traditionally-fermented foods, usually containing fish or meat (Lindström *et al.*, 2006; Peck, 2006; Peck, 2009).

The inability of Group II *C. botulinum* strains to utilize complex protein from milk or meat as an energy source (Holdeman & Brooks, 1970) is the basis for their denomination as non-proteolytic and this characteristic is employed when distinguishing them from proteolytic strains on casein based agars. However, several *C. botulinum* type E strains have been shown to possess some proteolytic activity as they are able to digest gelatin (Nakane & Iida, 1977). In contrast to Group I, growth of non-proteolytic *C. botulinum* strains depends on carbohydrates; they are able to ferment a wide range of carbohydrates like fructose, maltose, mannose, and sucrose (Holdeman & Brooks, 1970; Carter & Peck, 2015) and are therefore considered saccharolytic. Acetate and butyrate are the main metabolites produced (Holdeman & Brooks, 1970; Peck, 2009). When studied by AFLP the genetic diversity within Group II *C. botulinum* was higher than within Group I (Keto-Timonen *et al.*, 2005), however, a recent study comparing a large number of *C. botulinum* genome sequences did not demonstrate a clear difference in the level of diversity of the two groups (Williamson *et al.*, 2016). Serotype E strains form a subset distinct from the non-proteolytic serotype B and F strains (Keto-Timonen *et al.*, 2005; Stringer *et al.*, 2013), which reflects to some degree in their saccharolytic activity (Stringer *et al.*, 2013).

Being psychotrophic, Group II *C. botulinum* has a commonly-assumed optimum growth temperature of about 25-30 °C (Lindström *et al.*, 2006; Peck, 2009; Carter & Peck, 2015), with growth temperature limits of 3 °C and 45 °C (Eklund *et al.*, 1967; Johnson, 2000; Peck, 2009; Peck *et al.*, 2011). Non-proteolytic *C. botulinum* strains are able to form toxin at temperatures as low as 3 °C after incubation for 5 to 6 weeks under otherwise optimal conditions (Eklund *et al.*, 1967; Graham *et al.*, 1997). As mentioned for Group I, Group II also shows strain variation with regard to growth temperature (Jensen *et al.*, 1987; Stringer *et al.*, 2013). Group II *C. botulinum* appears to be less resistant to environmental stresses than Group I, with a water activity below 0.97 generated by 5% NaCl in the water phase or a pH below 5 prevent growth (Segner *et al.*, 1966; Peck *et al.*, 2011).

### 2.1.2. Botulinum neurotoxin

**Botulinum neurotoxin.** The German physician Justinus Kerner was the first to describe BoNT as a poison in 1822: he proposed a “sausage poison”, or “fatty acid” derived from spoiled sausages, to be the cause of a potentially-lethal paralytic illness he was confronted with in his practice (Kerner, 1822; Erbguth & Naumann, 1999; Erbguth, 2004). He concluded from his observations and experimental studies that the toxin is produced under anaerobic conditions in spoiled sausages, that it is a biological substance, which affects motor neurons, and that it is very strong, lethal in even small doses. In 1896, van Ermengem was able to associate the poisoning to an anaerobic bacterium he isolated and he called the organism “*Bacillus botulinus*”, which was later renamed to *C. botulinum* (Ermengem, 1897; Erbguth, 2008). Today it is known that BoNT produced by *C. botulinum* and some strains of *C. butyricum* and *C. baratii* is one of the most toxic biological substances known to mankind: it is estimated that less than 100 ng can cause human death (Wright, 1955; Gill, 1982; Schantz & Johnson, 1992; Arnon *et al.*, 2001; Peck, 2009). It is widely used in medical science as a therapeutic agent to treat neuromuscular disorders and in the cosmetic industry to reduce wrinkles (Schantz & Johnson, 1992; Mahant *et al.*, 2000; Bigalke, 2013). But it also has a high potential to be abused as a bioterrorism agent and is therefore listed as a Category A critical biological agent by the U.S. Centers for Disease Control and Prevention (Centers for Disease Control and Prevention, 2000; Arnon *et al.*, 2001).

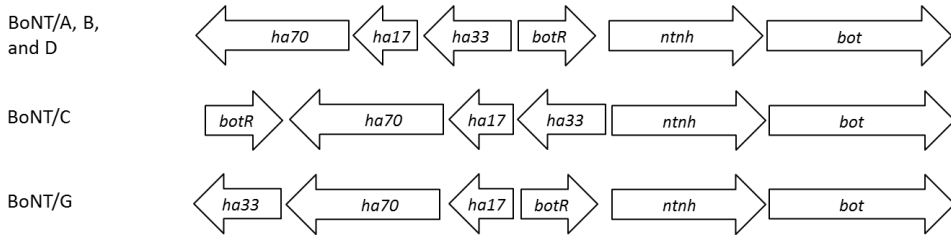
In 1919, after distinct serological properties of BoNTs from different *C. botulinum* isolates had been recognized, Burke designated BoNT serotypes A and B (Burke, 1919b). Antitoxin derived from animals immunized with BoNT serotype A (BoNT/A) did not protect against a number of the strains she studied; she named the heterologous toxin BoNT/B. To date seven different types of BoNT (A-G) have been identified. Recently, there has been a lively discussion about the discovery of a BoNT/H, which has finally been shown to be hybrid of serotypes A and F that can be fully neutralized by serotype A antitoxin in high doses or a combination of serotype A and F antitoxins (Barash & Arnon, 2014; Dover *et al.*, 2014; Johnson, 2014; Kalb *et al.*, 2015; Maslanka *et al.*, 2015; Fan

*et al.*, 2016). Modern molecular biological methods allow a more precise assessment of the different serotypes, as many BoNT protein sequences as well as neurotoxin-coding gene sequences are available and have been analyzed (Hill & Smith, 2013). The different BoNT serotypes can differ from ~25-45% in nucleotide sequence and ~37-70% in amino-acid sequence. Analysis of BoNT coding sequences in relation to the genetic background of *C. botulinum* strains led to the conclusion that BoNT genes have been introduced into the genome by horizontal gene transfer (Hill *et al.*, 2007; Hill *et al.*, 2007; Carter *et al.*, 2009). Knowledge of the sequences of the different BoNT coding genes further led to the development of a number of rapid PCR-based detection methods for *C. botulinum* with the ability to differentiate strains with different toxin serotypes (Franciosa *et al.*, 1994; Hielm *et al.*, 1996; Lindström *et al.*, 2001; Lindström & Korkeala, 2006; Fach *et al.*, 2009; Kirchner *et al.*, 2010).

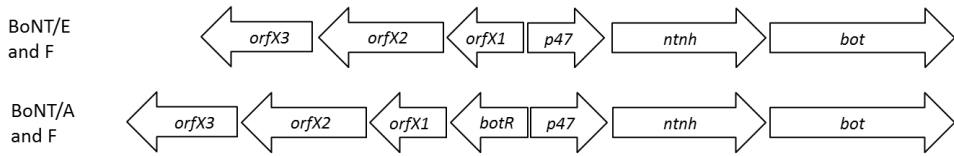
Further sequence variation exists within the serotypes of BoNT, which can have impact on antibody binding and neutralization as well as detection (Smith *et al.*, 2005; Hill & Smith, 2013). BoNTs of one serotype with a sequence variation of 2.5% at the amino acid level are commonly considered to be different subtypes (Smith *et al.*, 2005; Carter *et al.*, 2009). However, many authors prefer to define a subtype by the genetic clade a BoNT clusters into (Chen *et al.*, 2007; Hill *et al.*, 2015). Of the BoNT serotypes relevant to human health, eight subtypes of BoNT/A (A1-A8), eight of BoNT/B (B1-B8), seven of BoNT/F (F1-F7), twelve of BoNT/E (E1-E12), and one BoNT/A-F hybrid (also discussed as serotype H) have been described (Hill *et al.*, 2015; Kalb *et al.*, 2015; Kull *et al.*, 2015; Maslanka *et al.*, 2015).

The BoNT is formed during vegetative growth of *C. botulinum* as a single chain polypeptide of a molecular weight of approximately 150 kDa, which is heat labile and can be destroyed by heating to 80 °C for 5 min (Wright, 1955). It naturally forms a progenitor toxin complex of 300 to 900 kDa with several associated non-toxic proteins (ANTPs) named hemagglutinin (HA) and non-toxic non-HA (NTNH) (Chen *et al.*, 1998). The genes coding for BoNT and ANTPs are localized in the neurotoxin gene cluster (NTC), which *C. botulinum* strains may carry in their genome and/or on a plasmid (Group I and II) or on a phage (Group III) (Brüggemann, 2005). However, not all strains carry HA coding genes, some possess instead *p47* and *orfX1-X3* genes in their NTCs (Chen *et al.*, 2007; Jacobson *et al.*, 2008; Connan *et al.*, 2013; Hill *et al.*, 2015). NTCs of strains carrying HA coding genes are denominated “*ha* cluster”, NTCs of strains carrying *orfX* genes are called “*orfX* cluster” (Fig.1).

### ha toxin clusters



### orfX toxin clusters



**Figure 1.** Simplified examples of some neurotoxin gene cluster arrangements

The BoNT is activated by extracellular proteolysis through host proteases or endogenous bacterial proteases, leading to the mature di-chain holotoxin consisting of a ~100 kDa heavy chain (HC) and a ~50 kDa light chain (LC) joined via a disulfide bond (DasGupta, 1989). The crystal structure of the BoNT/A has been solved and contributes to the understanding of the mode of action of BoNT (Lacy *et al.*, 1998). Early studies identified BoNT toxicity to be related to acetylcholine (ACh) (Torda & Wolff, 1947; Burgen *et al.*, 1949). The release of the neurotransmitter ACh at the neuromuscular junction is prevented by BoNT, leading to a pre-synaptic block, thus disrupting signal transduction. BoNT needs to perform a series of steps to achieve this (Simpson, 1980; Simpson, 2013). In cases of oral intoxication, BoNT crosses the epithelial cell barrier of the gastrointestinal tract through an endocytosis-dependent mechanism to enter the bloodstream prior migration to its target cells, the motor neurons (Couesnon *et al.*, 2008; Connan *et al.*, 2015). The subsequent interaction of BoNT with motor neurons has been intensively studied and recently thoroughly reviewed (Aoki & Guyer, 2001; Poulain *et al.*, 2008; Binz, 2013; Fischer, 2013). Briefly, BoNT first needs to enter the neuron, which takes place by receptor-mediated endocytosis. The C-terminal binding domain of the HC interacts with the cell surface by binding to a surface ganglioside and a glycoprotein. In the acidic environment of the endosome, the HC N-terminal translocation region then forms a cation selective protein-conducting channel, which allows translocation of the unfolded LC into the cytosol. There the LC releases from the HC, refolds and can establish its zinc-dependent endopeptidase activity. The protease specifically cleaves soluble N-ethylmaleimide-sensitive factor attachment protein receptors (SNARE), which

are important for neurotransmitter exocytosis. The LC of the different serotypes have been shown to be substrate specific; BoNT/A, C, and E cleave synaptosomal associated protein of 25 kDa (SNAP-25), whereas BoNT/B, D, F, and G cleave the synaptic integral vesicle associated membrane protein (VAMP), also called synaptobrevin. BoNT/C is additionally able to cleave syntaxin, another synaptic membrane SNARE protein. The cleavage site within the SNAREs is also serotype specific, resulting in cleavage products of different sizes. This serotype specificity has been utilized to develop endopeptidase assays for sensitive BoNT detection (Hallis *et al.*, 1996; Jones *et al.*, 2008). Regardless of target protein and cleavage site, the activity of BoNT in the motor neuron prevents binding of the synaptic vesicles containing ACh and therefore inhibits release of this neurotransmitter into the neuromuscular junction. This disruption of ACh release from motor neurons leads to the typical clinical sign of botulism, flaccid paralysis without loss of consciousness.

Even though BoNT can be lethal, its potential for medical use has been part of botulinum research from the beginning as already Kerner 1822 carefully hypothesized the application of BoNT to treat neurological diseases (Kerner, 1822; Erbguth & Naumann, 1999; Erbguth, 2004). A big step towards the therapeutic use of BoNT was the development of a method to concentrate and crystallize BoNT/A by precipitation, although this research was originally intending the development of an efficient bioweapon (Lamanna *et al.*, 1946; Scott, 2004). Nevertheless, purified BoNT/A provided the basis for all studies of the clinical use of the bacterial neurotoxin. Initially BoNT/A was used experimentally to treat strabismus in monkeys and later humans by local injections into the eye muscles (Scott, 1980). But the ability of BoNT to disrupt signal transduction at the neuromuscular junction, thus to paralyze selected muscles when locally administered, is today widely used to treat a number of diseases like strabismus, dystonia, cerebral palsy, blepharospasm, torticollis, and other muscular disorders (Schantz & Johnson, 1992; Mahant *et al.*, 2000; Scott, 2004). Further BoNT/A can be used cosmetically to treat wrinkles, which has developed into a major business. Lately the toxin's potential analgetic effect is a new focus in clinical BoNT research, as it has shown to be effective for those suffering chronic neuropathic pain and for migraine treatment (Silberstein *et al.*, 2000; Cui *et al.*, 2004; Ranoux *et al.*, 2008). Despite its status as the most toxic naturally-occurring substance, BoNT is considered to be a safe therapeutic (Naumann & Jankovic, 2004; Bigalke, 2013).

**Regulation of BoNT expression.** BoNT expression is a highly energy consuming mechanism for *C. botulinum*, therefore its regulation can be expected to be tightly controlled in the bacterium. Full characterization of this regulation would be a significant contribution to long-term aims of botulinum research: control and prevention of BoNT production in food and the human body, as well as improvement of BoNT quality and production for medical use. During vegetative growth in batch cultures the expression of BoNT is growth-phase dependent and begins at the onset of exponential growth with a strong increase during late exponential growth and transition phase (Bradshaw *et al.*,

2004; Couesnon *et al.*, 2006; Chen *et al.*, 2008). The transcription of the NTC follows this pattern, peaking in the transition phase. Growth of *C. botulinum* and BoNT expression are affected by environmental conditions like nutrient availability, environmental pH, salt, temperature and other factors, which have been intensively studied to improve culture conditions to enhance toxin yield in (industrial) fermentations as well as to understand its role as a foodborne pathogen (Bonventre & Kempe, 1959a; Bonventre & Kempe, 1959b; Bonventre & Kempe, 1960; Segner *et al.*, 1966; Eklund *et al.*, 1967; Siegel & Metzger, 1979; Siegel & Metzger, 1980; Whitmer & Johnson, 1988; Couesnon *et al.*, 2006). With modern molecular biological tools becoming available, a growing number of publications try to elucidate the molecular bases of the regulation of BoNT expression, as recently reviewed by Connan *et al.* and Carter *et al.* (Connan *et al.*, 2013; Carter *et al.*, 2014).

A major step in understanding the molecular mechanisms of BoNT regulation was the discovery of BotR, a positive regulator of NTC genes in *C. botulinum* type A (Hauser *et al.*, 1994; Marvaud *et al.*, 1998). BotR, being considered the key regulatory factor for NTC transcription, is an alternative sigma factor, highly related to TetR of *C. tetani*; it binds as a subunit to RNA polymerase core enzyme and promotes transcription of the NTC operon genes by -35 and -10 region recognition in its target promoters (Raffestin *et al.*, 2005). Interestingly, serotype E and some type F strains lack the *botR* gene in their NTC operon (Chen *et al.*, 2007; Dover *et al.*, 2011).

Two-component signal transduction systems (TCSs) are specialized mechanisms to sense the bacterial environment and induce a subsequent response. They consist of a membrane-located sensor histidine kinase and a cytoplasmic response regulator, usually a DNA-binding protein that regulates target gene expression. An environmental stimulus leads to autophosphorylation of the sensor that then phosphorylates its specific response regulator, which typically changes its DNA binding activity. As TCSs have been shown to play a role in virulence of many bacteria (Beier & Gross, 2006), their relation to BoNT/A expression has recently been systematically studied in *C. botulinum* (Connan *et al.*, 2012; Zhang *et al.*, 2013). Using the mRNA antisense method, it was discovered that at least three TCSs positively control BoNT/A expression in a *botR*-independent manner (Connan *et al.*, 2012). Further, it was demonstrated that the TCS system CBO0787/CBO0786 directly suppresses expression of the BoNT/A, as well as ANTP coding genes; it was shown that the response regulator CBO0786 binds to promotor regions in the NTC (Zhang *et al.*, 2013). Thus far, this is the only discovered negative regulator of BoNT/A synthesis.

The association of BoNT expression to the availability of certain nutrients in the bacterial environment was described during early investigations into *C. botulinum* (Bonventre & Kempe, 1959a; Boroff & DasGupta, 1971; Patterson-Curtis & Johnson, 1989). Control of BoNT synthesis might therefore be tightly linked to nutritional signals and metabolic pathways, as are many virulence factors in other pathogenic bacteria (Dineen *et al.*, 2007; Richardson *et al.*, 2015). It has been recently shown that CodY, a global regulator of the transition from exponential to stationary phase, positively regulates BoNT gene expression in *C. botulinum* (Zhang *et al.*, 2014). CodY protein is thought to directly regulate BoNT expression, as it interacts in a guanosine-triphosphate (GTP)

dependent manner with the BoNT gene promotor region. The enhancement of CodY binding to the promotor region by GTP led the authors to the conclusion, that CodY-regulated BoNT expression is linked to the nutritional status of the cell, reflecting in intracellular GTP concentration, and that it might be associated to pyruvate metabolism (Zhang *et al.*, 2014).

Since BoNT expression is growth-phase dependent, an association to cell density seems likely. Quorum sensing is a process that allows bacteria to monitor the cell density by the means of signaling molecule concentration in their environment and to adjust their cellular behavior in accordance to it (Waters & Bassler, 2005). It is involved in the regulation of virulence in different bacteria (Winzer & Williams, 2001). In *C. botulinum*, the *agr-2* locus, coding for proteins related to the *Staphylococcus aureus* *agr* quorum sensing system, has been linked to the regulation of BoNT expression, as its inactivation resulted in decreased BoNT/A levels (Cooksley *et al.*, 2010).

### 2.1.3. Botulism

Botulism is a rare, often severe neuroparalytic disease characterized by flaccid paralysis due to disruption of signal transduction at the neuromuscular junction caused by BoNT intoxication in mammals and birds. Typical symptoms often start with blurred vision, difficulties to swallow and speak, followed by descending paralysis and muscle weakness; decrease of lacrimation and secretion from the salivary glands, gastrointestinal and bladder paralysis are also commonly described (Sobel, 2005; Erbguth, 2008; Peck, 2009). If untreated, flaccid paralysis of respiratory and cardiac muscles might lead to death. Differential diagnoses include Guillain-Barré syndrome, Miller-Fisher syndrome, chemical intoxication, and stroke. Since only BoNT circulating in the bloodstream can be neutralized by intravenous injection of specific antitoxin, the treatment of the disease often relies on intensive additional palliative care, in very severe cases mechanical ventilation.

Recovery from the disease takes a long time. The function of paralyzed synapses is temporally replaced by newly sprouting cells before the activity in the parental neurons is restored (Meunier *et al.*, 2003). The time until full recovery is dependent on the BoNT serotype and dose and may take a couple of weeks to several months, cases requiring mechanical ventilation for more than a year have been reported (Sheth *et al.*, 2008). Different forms of botulism have been described, and even though their clinical symptoms are very similar, they differ in their pathogenesis (Sobel, 2005). The classical foodborne botulism is intoxication with BoNT formed by vegetative *C. botulinum* in food. In contrast, wound, infant, and intestinal botulism are toxico-infections, infections with *C. botulinum* forming BoNT in the human body. Further the iatrogenic botulism after therapeutic and cosmetic use of BoNT (Bakheit *et al.*, 1997; Chertow *et al.*, 2006) and potential inhalational botulism (Park & Simpson, 2003), both intoxications, have been described. Botulism in animals is usually the result of intoxication with BoNT and resembles foodborne botulism. However, *C. botulinum* may also colonize the animal's intestine. If this intestinal form is caused by strains of Group I or Group II, it may be of

concern for human health if food products derived from an infected animal get contaminated (Lindström *et al.*, 2010). The most common forms -foodborne, infant and wound botulism- are described here in more detail.

**Foodborne botulism.** Intake of BoNT formed by toxigenic *C. botulinum* culture in food leads to foodborne botulism, a food poisoning, which has been traditionally the most prevalent form of botulism and is therefore called “classical” botulism. It was also the first form of the disease described in 1817 by Steinbruch and Kerner (Erbguth, 2004). The name botulism relates to the first food item observed to induce the disease, spoiled blood sausage (from the Latin word *botulus*, meaning “sausage”). However, BoNT formation in foods can precede signs of spoilage, thus seemingly unspoiled food items may indeed contain high levels of BoNT (Lawlor *et al.*, 2000; Kasai *et al.*, 2005; Lindström *et al.*, 2006). Foodborne botulism is the most prevalent form of botulism in Europe, and expected to be underreported (Therre, 1999; Peck *et al.*, 2011; Carter & Peck, 2015). Cases are often linked to the consumption of insufficiently processed, canned or bottled, often home-preserved foods, (Group I *C. botulinum*), traditionally fermented or smoked meat or fish products (primarily Group II), or inadequately stored food items (temperature abused, beyond due-date) (Group I and II) (Lindström *et al.*, 2006; Peck, 2009; Cowden, 2011; Carter & Peck, 2015). Modern food processing methods like mild pasteurization treatments, anaerobic packaging, extended shelf lives and chilled storage have led to an increase in Group II *C. botulinum* caused outbreaks related to commercial products (Lindström *et al.*, 2006; Peck, 2006).

**Infant botulism.** The premature intestines of children less than one year of age can be colonized with *C. botulinum* after ingestion of clostridial spores due to lack of competitive intestinal microbiota. Synthesis of BoNT *in vivo*, followed by absorption into the bloodstream, can lead to infant botulism (Arnon *et al.*, 1981). Typical clinical signs are poor feeding and lethargy, constipation, hypotonia, dilated pupils, and absent reflexes in infants. Group I BoNT/A or B producing strains are usually involved, however, cases caused by BoNT/F or E forming *C. botulinum* as well as BoNT/F forming *C. baratii* and BoNT/E forming *C. butyricum* have also been reported (Koepke *et al.*, 2008). The disease has been associated with sudden infant death syndrome. Spores of *C. botulinum* present in honey or dust have been identified as the primary source for the infection of infants, but also aquatic reptiles in the household have been linked to the disease (Arnon *et al.*, 1981; Nevas *et al.*, 2002; Derman *et al.*, 2014; Shelley *et al.*, 2015). After suffering from infant botulism, clinically healthy children can carry and excrete vegetative cells and spores of *C. botulinum* for several months and need therefore to be considered a potential source of infection for caretakers and other children (Derman *et al.*, 2014). The infant form is the most prevalent form of botulism to date in the United States, however, it is believed to be strongly underreported (Koepke *et al.*, 2008). A human-derived immunoglobulin was developed for the treatment of infant botulism to reduce adverse effects caused by the



administration of antitoxin produced in animals and has proven to be beneficial for recovery of young patients (Arnon *et al.*, 2006).

**Wound botulism.** Contamination of deep wounds with spores of *C. botulinum* can result in vegetative growth and *in situ* BoNT synthesis in the anaerobic wound environment. Entrance of the toxin into the human bloodstream can lead to systemic intoxication. This rare form of botulism has so far been related mainly to environmental contamination of traumatic injuries (Werner *et al.*, 2000; Sobel, 2005; Schroeter *et al.*, 2009). It has recently become an increasing problem in drug abusers, especially those injecting paravenously, due to contaminated heroine and needles. The clinical appearance resembles foodborne botulism, beside the lack of gastrointestinal symptoms. In addition to the conventional botulism treatment, antimicrobial therapy is indicated and the infected wound needs to be cleaned (Sobel, 2005).

## 2.2. Relevance of *C. botulinum* for the food industry

The costs of treating foodborne botulism have been estimated to be £22,000 (today worth about €52,000) per patient in an outbreak linked to contaminated hazelnut yoghurt produced in the United Kingdom in 1989 (Roberts, 2000). Other outbreak-related costs arise from outbreak investigations (estimated £6,000 in the above-mentioned case), recall of the product, loss of consumer trust, and possible legal charges of affected people. In the U.S., there are lawyer's offices specialized in representing victims of foodborne illnesses, including botulism (e.g. [www.botulismblog.com](http://www.botulismblog.com)). An outbreak in the U.S. from 1978 involving 34 people was quoted to have created costs of more than \$5,000,000; in 1997 estimations were made of costs up to \$30 million per human foodborne botulism case caused by a commercial product (Mann *et al.*, 1983; Setlow & Johnson, 1997). Even though outbreaks related to commercial foods are relatively rare, they cause severe disease, might affect a large number of people, and have a dramatic economic impact on the health care system as well as the company involved, potentially leading to bankruptcy of the business (Mann *et al.*, 1983; Setlow & Johnson, 1997; Sobel *et al.*, 2004; Lindström & Korkeala, 2006; Carter & Peck, 2015).

Since *C. botulinum* spores are ubiquitous in the environment, contamination of raw materials in food production can never be entirely excluded, even when applying good hygiene practice to keep contamination levels low. Therefore effective measures to control and prevent formation of toxigenic culture in food products are indispensable. Botulism outbreaks related to commercial products can often be linked to process failure, post-process contamination, use of contaminated ingredients, and temperature abuse, as well as other improper food handling practices by the consumer (Sobel *et al.*, 2004; Lindström & Korkeala, 2006; Lindström *et al.*, 2006).

The differences between Group I and Group II *C. botulinum* regarding physiology and especially spore resistance have a great impact on their risk profiles (Lindström *et al.*,

2006; Peck, 2009; Carter & Peck, 2015). This has to be addressed by the food industry. Group I strains are mesophilic and not able to grow under 10 °C, therefore outgrowth and BoNT synthesis of these strains can be easily controlled by chilled storage. However, as their highly-resistant spores might survive even strong heat treatment, they pose a risk in products preserved by canning or bottling, which are intended to be stored for extended time at room temperature under anaerobic conditions. Group I *C. botulinum* is commonly related to home-canned non-commercial products, since introduction of harsh heat treatment during the industrial canning process (the so-called botulinum cook, 3-6 min at 121 °C) has greatly reduced the number of cases related to commercial products (Stumbo *et al.*, 1975; Peck, 2009; Setlow & Johnson, 2013; Dahlsten *et al.*, 2015). However, insufficient heat treatment and post processing contamination have resulted in several outbreaks linked to industrial products, some of them leading to human death (Sobel *et al.*, 2004; Peck, 2009; Jalava *et al.*, 2011). A major problem that remains is improper food-handling practice, resulting in temperature abuse on a consumer as well as retail level, and insufficient heating before consumption. Room temperature storage of non-acidified products, which did not undergo heat treatment sufficient to kill heat resistant spores, allows growth and BoNT production of *C. botulinum* Group I. Some of the products involved in outbreaks in the past years were found to be labelled insufficiently with regard to storage temperature (Sobel *et al.*, 2004).

In recent years, the availability of refrigerated processed foods of extended durability (REFPED) has grown to meet consumers' increasing demand for convenient, mildly-treated food with high gustatory quality (Lindström *et al.*, 2006; Lindström *et al.*, 2006; Peck, 2006; Peck *et al.*, 2008). These products contain low levels of salt and other preservatives and are only minimally heat treated, but nevertheless have long shelf lives. Thus, the hurdles commonly used to ensure safety of food products, like heat treatment, increased osmolarity, or low water activity, low pH, and preservatives, are kept low and might be insufficient. Therefore, the control of microbial growth and BoNT formation in these products relies to a substantial degree on storage at chilled temperatures (Leistner, 2000; Lindström *et al.*, 2006; Peck, 2006). This measure is sufficient to prevent risk of Group I *C. botulinum*. However, being psychrotrophic, Group II is capable of growth and BoNT formation at chill temperature, especially during extended shelf life achieved by modified atmosphere packaging (Eklund *et al.*, 1967; Lindström *et al.*, 2006). Further, competitive microbial population in the product is inhibited by lack of oxygen or killed by the applied mild heat treatment, which in turn might be survived by clostridial spores (Hyttia-Trees *et al.*, 2000). In addition, many REPFEDs are intended not to be heated prior consumption. These factors increase the risk of foodborne botulism caused by REPFEDs and also explain the increase of botulism cases associated to commercial products related to Group II strains paralleling the emergence of REPFEDs (Sobel *et al.*, 2004; Lindström *et al.*, 2006; Peck, 2006; Peck, 2009). Many of these cases were linked to the consumption of raw, cured, or smoked fish and sea food (Korkeala *et al.*, 1998; Lindström *et al.*, 2006; King *et al.*, 2009). These products are of special concern as high levels of Group II *C. botulinum* spores are commonly reported in their raw materials

(Hielm *et al.*, 1998b; Hyytiä *et al.*, 1998). Many studies have been performed and predictive models were developed to estimate the effect of heat treatment and food materials and ingredients on growth from Group II spores during chilled storage in REPFEDs, in order to help to control the risk of foodborne botulism by Group II *C. botulinum* (Hyytiä-Trees *et al.*, 2000; Lindström *et al.*, 2006; Peck, 2006; Peck *et al.*, 2008; Ihekweba *et al.*, 2016; Ihekweba *et al.*, 2016).

Even though the hazards regarding BoNT production in commercial food products have been recognized and risk management strategies have been developed and implemented for decades, the control of *C. botulinum* remains an enormous challenge for the food industry (Lindström *et al.*, 2006; Ihekweba *et al.*, 2016).

### **2.3. Heat stress response in the Gram-positive model organism *Bacillus subtilis***

Being constantly exposed to changing and challenging environments, bacterial survival depends substantially on the ability to immediately respond to growth-limiting stresses. The impact of temperature, one of the most important environmental factors affecting all living cells, on the Gram-positive model organism *B. subtilis*, but also on human pathogens like *Listeria monocytogenes*, *C. difficile*, and *Staphylococcus aureus*, has been studied intensively in the past years (Hecker *et al.*, 1996; Helmann *et al.*, 2001; Schumann, 2003; Anderson *et al.*, 2006; van der Veen *et al.*, 2007; Ternan *et al.*, 2012). The response to sudden temperature increase leads to a shift in the cellular behavior on both a transcriptional and a translational level, the so-called "heat shock response", in order to enhance survival (Schumann, 2003). In pathogenic bacteria modulation of virulence factors has been observed in response to heat stress (Anderson *et al.*, 2006; van der Veen *et al.*, 2007; Emerson *et al.*, 2008). Studies on the heat shock response of bacteria are of particular value as it resembles the general stress response to a large degree, which is utilized to resist other environmental challenges like salt, pH, or ethanol stress, and therefore presents a model for it (Hecker *et al.*, 1996). Especially foodborne pathogens encounter a number of different stresses during processing, storage, but also in the human host, one of them is heat stress.

An increase of only a few degrees in environmental temperature initiates a rapid heat shock response in the bacterial cell. The assumed reason for this seemingly low threshold is the significant temperature sensitivity of protein conformation and stability (Richter *et al.*, 2010). At elevated temperature, a proportion of the intracellular proteins will be misfolded, unfolded, even denatured, or aggregated, and overall cellular function will be impaired (Baldwin, 1986; Dobson, 2003). But the damaging effect of heat is not limited to proteins. Other cellular components like rRNA, ribosomes, DNA, and the cell membrane will also be affected by heat (Lim & Gross, 2011). It has been commonly accepted that the increasing amount of misfolded or unfolded protein in the cytosol is the signal that triggers the heat shock response in bacteria, since one main aim of the heat shock response is to

preserve protein homeostasis (Lim & Gross, 2011). But recently it has been discovered that one major regulator of the bacterial heat shock response, the transcriptional repressor CtsR, binds in a temperature-dependent manner to DNA and acts itself as a thermosensor (Elsholz *et al.*, 2010). In *B. subtilis*, genes coding heat shock response proteins are grouped according to their mode of regulation. Its heat shock response is dominated by three major regulons: the SigB, HrcA, and CtsR controlled regulons (Hecker *et al.*, 1996; Schumann, 2003).

The largest group of proteins induced immediately after heat shock in *B. subtilis* are considered to be general stress proteins; they are also activated by other stresses like salt, ethanol, desiccation, acidity, or antibiotics, and under energy stress due to starvation for oxygen, glucose, or phosphate (Hecker *et al.*, 1996; Schumann, 2003; Hecker *et al.*, 2007). The more than 150 genes coding these proteins, the Class II heat shock genes (HSG), are under positive control of an alternative sigma factor, SigB (Helmann *et al.*, 2001; Price *et al.*, 2001; Price, 2011). The SigB regulon is rapidly activated after heat shock in *B. subtilis*, but the expression decreases from 10 min after a temperature up-shift (Schumann, 2003). A number of the genes code for proteins with clear protective functions: several are related to reduction of oxidative stress, DNA binding and DNA protection, transporters for compatible solutes, antibiotic resistance, cold stress resistance, and cell envelope functions (Hecker *et al.*, 2007; Price, 2011). However, the SigB regulon encompasses predominantly genes with so far unknown functions. The regulon plays a crucial role with regard to virulence in *L. monocytogenes* and *S. aureus* (Anderson *et al.*, 2006; van der Veen *et al.*, 2007). It is believed that no *sigB* homologs are present in any anaerobic bacteria (Hecker *et al.*, 2007). Several genes originally annotated as *sigB* in clostridial strains (Sebahia *et al.*, 2006; Fonknechten *et al.*, 2010; Bao *et al.*, 2011) were later re-annotated as RNA polymerase sigma-70 factor coding genes. For *C. botulinum*, a *sigB* homolog has never been described, nevertheless, homologs to some *B. subtilis* Class II HSGs are present in its genome (Sebahia *et al.*, 2007).

The two other major classes of *B. subtilis* HSGs, Class I and Class III, code for proteins primarily addressing the challenge of protein misfolding, denaturation, and aggregation in the heat-stressed bacterial cell. Both classes of HSGs are present in *C. botulinum*. They encode a protein quality control system of molecular chaperons and proteases that is involved in protein folding, refolding, control, and degradation. Both classes are under negative transcriptional regulation and de-repressed by non-native proteins.

The Class I HSGs code for molecular chaperones. These proteins assist misfolded, as well as newly synthesized unfolded proteins to reach their native state, and therefore promote proper protein assembly (Langer *et al.*, 1992a; Schröder *et al.*, 1993; Hartl & Hayer-Hartl, 2002). Class I HSGs consist of two operons, the *dnaK* and the *groELS* operon, which are universally conserved through all kingdoms. In Gram-positive bacteria like *B. subtilis*, they are under the control of the transcriptional repressor HrcA (Schulz & Schumann, 1996; Schulz & Schumann, 1996; Schumann, 2003). Their expression involves a SigA-dependent promoter.

Non-native proteins expose a high ratio of hydrophobic amino acid residues on their surface. The molecular chaperonin GroEL forms a cylinder of 14 subunits, which complexes with its co-chaperonin GroES (Langer *et al.*, 1992b; Hayer-Hartl *et al.*, 2015). This cylinder provides an isolated, highly hydrophilic environment triggering protein folding. It binds unfolded protein and encapsulates it, supporting burying of hydrophobic residues to the inside of the protein and thus mediating correct folding (Martin *et al.*, 1993). The binding affinity of proteins to the GroEL chaperonin is ATP controlled. In *B. subtilis* GroEL plays a major role in response to various stresses, but also in de novo protein folding (Hartl, 1996; Hartl & Hayer-Hartl, 2002; Dobson, 2003). The DnaK chaperone, encoded in the second HrcA regulated operon, also binds and stabilizes unfolded regions of proteins, keeping them separated from unspecific interaction partners, thus reducing undesired irreversible protein aggregation and misfolding (Langer *et al.*, 1992a). The molecular chaperone DnaJ is also *dnaK*-operon encoded and is thought to stabilize the complex of non-native protein and DnaK. The unfolded protein is then transferred with the help of GrpE, also *dnaK*-operon encoded, to the GroELS complex for refolding (Langer *et al.*, 1992a). In the Gram-negatives *E. coli* and *Thermus thermophilus*, it has been shown that DnaK is further able to repair heat-induced protein damage and aggregation in cooperation with the proteins GrpE and DnaJ, and the Class III HSG-encoded ClpB protease (Schröder *et al.*, 1993; Motohashi *et al.*, 1999). Class I HSGs are under negative control of HrcA. This transcriptional repressor needs to be continuously refolded by the GroELS complex to remain active. High temperature stress leads to an increase of unfolded proteins in the cytosol, which will compete with HrcA for binding to GroELS and eventually titrate it away (Mogk *et al.*, 1998). Inactivation of HrcA then will lead to de-repression of Class I HSGs.

Irreversibly-damaged proteins need to be removed from the bacterial cell, even though refolding of non-native proteins induced by heat stress is the preferred mechanism by any organism as it is less energy consuming. Proteolytic enzymes contribute to the heat shock response by clearing up these denatured proteins via degradation. The Class III HSGs of *B. subtilis* are organized into three operons and code for ATP-dependent Clp proteases; *clpP* for the proteolytic, *clpC* and *clpE* for the ATPase subunits (Schumann, 2003). ClpC and ClpP have also been associated to the stationary-phase regulatory network and the development of competence in *B. subtilis* (Msadek *et al.*, 1994; Msadek *et al.*, 1998). Clp proteases are considered to be the major protein quality control system for the cell and are under negative regulation of CtsR (Derré *et al.*, 1999; Schumann, 2003). This proteolytic system is strictly regulated: CtsR is inactivated by the kinase McsB, which again is inactive when bound to the ClpCP complex. However, this complex has a higher affinity to unfolded proteins, which if present in high concentration leads to release and activation of McsB after heat shock. This will result in inactivation of the transcriptional repressor CtsR and finally to activation of Class III HSGs (Kirstein *et al.*, 2005). CtsR itself binds to DNA in a temperature-dependent manner, with reduced binding at high temperature, and has been considered to be a thermosensor (Elsholz *et al.*, 2010).

The exposure to sublethal heat stress has been shown to have a protective effect on subsequent heat treatment at lethal temperatures in *B. subtilis* (Volker *et al.*, 1992). Activation of the heat shock response induced a preadaptation of the bacterial cell, increasing its thermotolerance and protecting it shortly after from otherwise lethal temperatures. In addition, this mild heat treatment caused so-called cross-protection, permitting the bacteria to survive otherwise toxic NaCl concentrations (Volker *et al.*, 1992). Mild NaCl stress also enhanced thermotolerance, although to a lesser degree. Stress-induced acquired thermotolerance and cross-protection have been reported for other foodborne pathogens such as *E. coli*, *L. monocytogenes*, and *Salmonella* and have been acknowledged as a risk with regards to food safety, especially when applying hurdle technology (reviewed by Wesche *et al.*) (Lou & Yousef, 1996; Juneja *et al.*, 1998; Wesche *et al.*, 2009).

## **2.4. Molecular mechanisms of stress response in *C. botulinum***

### **2.4.1. Heat stress response**

One of the first studies investigating the heat shock response in *C. botulinum* observed an overexpression of nine heat shock proteins when mid-exponentially growing batch culture of the type A strain Hall was exposed to heat shock from 37 °C to 45 °C (Shukla & Singh, 1999). The overexpression of these proteins was detected from 15 min after heat shock onwards and reached a maximum after 30 min. The bacterial culture further showed a change in growth behavior: 4 h post heat shock a lower cell density at 45 °C compared to 37 °C was recorded, which then dramatically dropped after 10 h. One of the overexpressed proteins was described as a DnaJ-like chaperone; the first heat shock protein to be reported in *C. botulinum* (Shukla & Singh, 1999). Another protein found to be overexpressed was later identified to be the ANTP HA33 (Shukla & Singh, 1999; Shukla & Singh, 2009). The heat shock chaperonin complex GroELS has been described in a *C. botulinum* type D strain after it was detected in culture supernatant during toxin production under growth conditions considered not stressful (Sagane *et al.*, 2003). The toxigenic type D strain showed higher GroELS expression compared to a non-toxic strain, which prompted speculation for a possible role of the chaperonin complex in toxin production or translocation over the cell membrane, which has not yet been confirmed (Sagane *et al.*, 2003).

A recent transcriptomic analysis of *C. botulinum* ATCC 3502 under heat stress at 45 °C for 15 min revealed overexpression of the Class I HSG operons *dnaK* and *groESL*. In contrast, Class III HSGs were found to be unaffected in *C. botulinum* (Liang *et al.*, 2013). The exposure of the mid-exponential growing culture to heat shock further led to reduced expression of genes related to aminoacyl-tRNA synthetase, ribosomal structure and cell division proteins, confirming a growth arrest after heat shock detected earlier (Shukla & Singh, 1999; Liang *et al.*, 2013). Other genes affected by heat stress in mid-exponential

cells were associated to transcriptional regulation, energy production and conversion, transport, cell wall and membrane biogenesis, and flagella biosynthesis. However, their contribution to the *C. botulinum* heat shock response remains to be characterized. Interestingly, no significant changes in the expression levels of any NTC genes or genes related to sporulation could be detected (Liang *et al.*, 2013). Only mild up-regulation was observed for *ha17* and for *ha33*, which codes for an ANTP considered by Shukla *et al.* as a heat shock protein (Shukla & Singh, 2009). In addition, comparison of *C. botulinum* culture growing at 37 °C with culture growing at 44 °C showed no significant differences in NTC gene expression (Couesnon *et al.*, 2006).

#### **2.4.2. Cold stress response**

The main effects of low temperature on cellular components are reduced membrane fluidity due to solidification of fatty acids, impairment of the transcriptional and translational machinery due to increased stability of secondary structures in nucleic acids, especially RNA, changes in protein folding and enzyme efficiency, and reduced ribosome function (Weber & Marahiel, 2003; Phadtare, 2004; Lim & Gross, 2011). Exposure to a sudden drop in environmental temperature leads to complex changes in the bacterial physiology to overcome these problems, the so-called cold shock response. In contrast to the bacterial heat shock response, the cold shock response appears to be regulated in a more heterogeneous manner and seems to employ a wider range of molecular systems (Weber & Marahiel, 2003). Given the importance of the foodborne pathogen *C. botulinum* with regard to growth and BoNT formation in food during storage at chill temperature, a number of studies have been performed to elucidate the molecular mechanisms of cold tolerance this bacterium utilizes (Söderholm *et al.*, 2011; Lindström *et al.*, 2012; Dahlsten *et al.*, 2014; Dahlsten *et al.*, 2014; Mascher *et al.*, 2014; Söderholm *et al.*, 2015).

Sequencing of the genome of the Group I *C. botulinum* strain ATCC 3502 revealed the presence of three genes annotated as cold shock genes *cspA* – *cspC*, containing a nucleic acid binding cold shock domain (Sebahia *et al.*, 2007). Of these, *cspB* has been shown to encode the major cold shock protein in the strain: its insertional inactivation led to the strongest growth impairment at low temperature (Söderholm *et al.*, 2011). In contrast, *cspA* inactivation led to no cold sensitive phenotype. However, the transcription of all three cold shock protein-coding genes was up-regulated 30 min after cold shock (Söderholm *et al.*, 2011). Further studies on Group I *C. botulinum* cold shock genes revealed the importance of *cspB* and *cspC* for other environmental stresses and motility (Derman *et al.*, 2015). Interestingly, cold shock protein-coding genes, even though thought to be universally conserved, appear to be absent in Group II *C. botulinum* type E strains (Söderholm *et al.*, 2013). It is unknown how their role in cold tolerance is compensated in these strains.

A recent study profiling the gene expression of the Group I *C. botulinum* strain ATCC 3502 after cold shock from 37 °C to 15 °C using DNA microarrays revealed an extensive remodeling of the bacterial metabolism after long term exposure to cold,

whereas the transcription of only 28 genes was affected 1 h after cold shock (Dahlsten *et al.*, 2014). The cold shock led to a slight up-regulation of *ha* genes, but left the rest of the NTC unaffected. Among the genes that were early up-regulated after cold shock was *csdA* (*cbo2802*), encoding a DEAD/DEAH box family RNA helicase. These types of helicases have been shown to be essential for *B. subtilis*. It has been proposed that they work in cooperation with cold shock proteins as chaperones to enhance the translation initiation of mRNA, which has increased stability at low temperature that is hampering ribosomal function (Hunger *et al.*, 2006). For *C. botulinum*, the *csdA* gene has been observed to be important for growth at low temperature, as its inactivation led to impaired growth and motility at 20 °C compared to 37 °C and increased low temperature growth limits (Söderholm *et al.*, 2015). The above-mentioned expression profiling study further observed a significant increase in the transcription levels of genes related to up-take of compatible solutes, which might function as cryo- and osmo-protectants in the bacterial cell (Dahlsten *et al.*, 2014). Prolonged exposure of the Group I *C. botulinum* strain ATCC 3502 also led to changes in the expression of genes related to fatty acid metabolism, probably leading to changes in the cell membrane composition to reduce its solidification, which is a problem commonly encountered during bacterial cold stress (Dahlsten *et al.*, 2014). Remodeling of the fatty acid composition in response to reduced growth temperature has been observed in Group II *C. botulinum* as well (Evans *et al.*, 1998). Other genes identified as cold stress affected in the microarray study could be related to, at least, redox stress, SOS response, changes in amino acid metabolism, and transcriptional regulation (Dahlsten *et al.*, 2014). However, their impact on cold tolerance in *C. botulinum* needs to be further characterized.

Not much is known about the regulatory network controlling the cold stress response of *C. botulinum*. Recently, TCSs sensing the bacterial environment have been shown to play a role in group I as well as Group II *C. botulinum* cold tolerance: Genes coding the for TCS CBO0366/CBO0365 and CBO2306/CBO2307 in the Group I strain ATCC 3502 (Lindström *et al.*, 2012; Derman *et al.*, 2013) and CLO3403/CLO3404 in the Group II strain E1 Beluga (Mascher *et al.*, 2014) were found to be up-regulated upon temperature down-shift and their inactivation led to a cold-sensitive phenotype with reduced growth performance at low, but not at optimal, temperature. Interestingly, an additional study further characterizing the CBO0365 response regulator revealed that the expression of its regulon was almost unaffected by temperature (Dahlsten *et al.*, 2014). However, the authors demonstrated that inactivation of a number of genes of this CBO0365 regulon led to deteriorated growth at suboptimal temperature. They therefore suggested that mechanisms controlled by the TCS CBO0366/CBO0365 related to the solvent formation pathway, components of the arsenic resistance machinery and phosphate uptake contribute to the cold stress response of Group I *C. botulinum* strain ATCC 3502 (Dahlsten *et al.*, 2014). None of these had previously been associated to temperature stress tolerance.

Another regulatory protein, the sporulation sigma factor SigK, has been shown to play a role in cold tolerance of Group I *C. botulinum* ATCC 3502 as well (Dahlsten *et al.*, 2013). The transcription of the SigK coding gene increased significantly after cold shock



and its inactivation led to reduced growth at low temperature. The authors speculated that SigK might to some degree substitute the *B. subtilis* general stress sigma factor SigB, which *C. botulinum* is lacking (Sebahia *et al.*, 2007; Dahlsten *et al.*, 2013).

### 2.4.3. Other stresses

Even though temperature-, especially cold-stress, is the most thoroughly studied environmental stress in *C. botulinum*, other stresses are also significant for this foodborne pathogen. In the environment, during food processing and storage, and also in the animal host, *C. botulinum* needs to resist changes not only in temperature but also in pH, osmolarity, water activity, nutrient availability, oxygen levels, and preservatives (Lindström *et al.*, 2006; Peck, 2009; Dahlsten *et al.*, 2015).

Several mechanisms associated to the cold shock response in Group I *C. botulinum* have been recently linked to the response to other stresses in the bacterium as well. The sporulation sigma factor SigK coding gene was found to be up-regulated after exposure to high NaCl concentration and its inactivation led to increase of lag time when grown at high salinity, whereas no effect on tolerance to acid stress was observed (Dahlsten *et al.*, 2013). An additional role for SigK in response and adaptation to hyperosmotic conditions was thus suggested. It has further been shown that the cold shock protein coding genes *cspB* and *cspC* are of importance for the response of *C. botulinum* to NaCl, low pH and ethanol stress (Derman *et al.*, 2015). Of these genes, the major cold shock gene *cspB* was again identified to have the greatest impact on the stress tolerance as its inactivation led to more deteriorated growth in the tested conditions. It was therefore proposed to have a universal role in stress response. Even though the inhibiting effects of many other environmental factors on the vegetative growth of *C. botulinum* have been thoroughly studied to improve food safety, their underlying molecular mechanisms have not yet been characterized.

The evident participation of cold tolerance mechanisms in the response to other stresses emphasizes that cross-protection and probably induced stress tolerance by sublethal stress most likely exist in *C. botulinum*. When using hurdle technology in food preservation to prevent growth and BoNT formation of *C. botulinum*, which is a common trend in the modern food industry, the applied mild treatments could hence lead to unexpectedly increased tolerance of subsequent preservation methods in minimally processed foods. Therefore, the effects and mechanisms of induced stress tolerance and cross-protection in *C. botulinum* require more thorough characterization and should be taken into consideration when designing measures to ensure microbial safety of foods with regard to *C. botulinum*.

### 3. AIMS OF THE STUDY

The objectives of this study were to explore the strain variation of Group I and Group II *C. botulinum* with regard to growth at stressful temperatures and to investigate mechanisms of heat shock and heat stress response of Group I *C. botulinum*.

The specific aims were:

1. To investigate the minimum and maximum growth temperatures of 23 Group I and 24 Group II *C. botulinum* strains and their growth rates at a low, optimal, and high temperature to explore strain variation (I, II),
2. To study the biological functions of the Class I heat shock genes *hrcA* and *dnaK* in the stress response of Group I *C. botulinum* strain ATCC 3502 (III), and
3. To obtain whole genome gene expression profiles for the Group I *C. botulinum* strain ATCC 3502 grown in continuous culture during heat shock, during and after adaptation to high temperature (IV).

## 4. MATERIALS AND METHODS

### 4.1. Strains and plasmids

Twenty-three Group I strains and twenty-four Group II strains were used to study strain variation with regard to growth temperature within the groups and were selected to represent strains with a wide genetic background and of different origins (I, II) (Table 2). The Group I strains included five strains of serotype A, two of type AB (one Ab and one A[B]), 14 type B, and two of type F. The Group II strains consisted of three serotype B, 16 type E, and five type F strains.

The Group I *C. botulinum* strain ATCC 3502 (III, IV) as well as mutant strains originating from this strain carrying a single insertional mutation in *hrcA* (*cbo2961*) or *dnaK* (*cbo2959*) (III), were used to examine mechanisms of high temperature stress tolerance. Cloning, donor, and mutant strains and plasmids are listed in Table 3.

**Table 2.** *Clostridium botulinum* wild type strains used in this study.

Type	Strain <sup>a</sup>	Origin	Source <sup>b</sup>
Group I <i>C. botulinum</i>			
A	ATCC 3502	Unknown	ATCC
	RS-3A	Pacific Red Snapper, USA, Pacific coast	S. Lindroth, UCDAVIS
	ATCC 25763	Type strain	ATCC
	DA 100/1 S	Danish honey	DFHEH
	96 A	Unknown	T. Roberts
AB	CDC 588	Botulism outbreak, USA	IFR
	NCTC 2916	Corn	CAMR
B	P 1/3	Canned deer meat, Finland	DFHEH
	M 1/3	Soil, Finland	DFHEH
	BL 150	Fish	Unilever
	BL 81/25	Asparagus	LFRA
	NCTC 3815	Cheese	Unilever
	DA 58/1	Danish honey	DFHEH
	M 316/5	Soil, Finland	DFHEH
	CDC 7827	Human stool	CDC
	M 193/15	Soil, Finland	DFHEH
	P 120/6	Household dust, infant botulism case, Finland	DFHEH
	ATCC 17841	Unknown	ATCC
	126 B	Unknown	Institut Pasteur
	525 S/8	Honey-comb pollen	DFHEH
	KS 44/10	Honey-comb pollen	DFHEH
F	ATCC 35415	Liver paste	P. McClure, Unilever
	ATCC 25764	Crab	ATCC

**Table 2.** Continued

Type	Strain <sup>a</sup>	Origin	Source <sup>b</sup>
Group II <i>C. botulinum</i>			
B	Eklund 17 (ATCC 25765)	Marine sediment	ATCC
	BL 93/6 (CDC 5900)	Human	IFR
	BL 90/4 (Prevot 59)	Unknown	ATCC
E	K3	Rainbow trout surface	DFHEH
	K8	Rainbow trout intestines	DFHEH
	K35	Vendace	DFHEH
	K36	Rainbow trout	DFHEH
	K42	Rainbow trout intestines	DFHEH
	K44	Rainbow trout intestines	DFHEH
	K51	Rainbow trout surface	DFHEH
	K101	Chub surface	DFHEH
	K117	White fish	DFHEH
	K119	Rainbow trout intestines	DFHEH
	K125	Rainbow trout roe	DFHEH
	BL 93/8 (CDC 8073)	Human	IFR
	31-2570	Unknown	DFHEH
	BL 81/26 (Beluga)	Beluga flipper	IFR
	S16	Fishpond sediment	DFHEH
	CB11/1-1	Whitefish roe	DFHEH
F	202 (ATCC 23387)	Marine sediment	ATCC
	BL 86/32 (Colworth 47)	Unknown	IFR
	BL 86/33 (Colworth 187)	Unknown	IFR
	BL 86/34 (Colworth 195)	Unknown	IFR
	610B8-6	Salmon	S. Lindroth, UCDAVIS

<sup>a</sup> multiple names of strains in brackets

<sup>b</sup>ATCC: American Type Culture Collection, Manassas, Va., USA; DFHEH: Department of Food Hygiene and Environmental Health, University of Helsinki, Finland; UCDAVIS: University of California, Davis, USA; IFR: Culture collection of the Institute of Food Research, Norwich, UK; CAMR: Centre for Applied Microbiology and Research, Salisbury, UK; LFRA: Leatherhead Food Research Association, Surrey, UK; CDC: Centers for Disease Control and Prevention, USA

## 4.2. Culture preparation (I-IV)

All *C. botulinum* strains used in the studies, with exception of the mutant strains (III), were cultured from spore stocks. These have been prepared from strains of the collection of the Department of Food Hygiene and Environmental Health, University of Helsinki, Finland, after incubation for 2-3 days on blood agar plates (5% bovine blood, 10 g/liter

agar), followed by re-inoculation of single colonies into 50 ml fresh tryptone-peptone-glucose-yeast extract (TPGY) broth (50 g/liter tryptone, 5 g/liter peptone, 20 g/liter yeast extract [Difco, BD Diagnostic Systems, Sparks, MD], 4 g/liter glucose [VWR International, Leuven, Belgium], 1 g/liter sodium thioglycolate [Merck, Darmstadt, Germany]) and incubation for 7 days. After the BoNT serotype of the cultures was confirmed by multiplex PCR (Lindström *et al.*, 2001), the cells were heat treated for 15 min at 85 °C in a water bath to kill remaining vegetative cells; the spores were washed and stored in sterile water at 4 °C. The *C. botulinum* mutant strains (III) were cultured from frozen stocks stored in TPGY broth supplemented with 20% glycerol (Sigma-Aldrich, Steinheim, Germany).

Routinely, the strains were initially grown from spore or frozen stocks for 24 h in 10 ml deoxygenated TPGY broth (I, II) or buffered TPGY broth (6.25 g/liter NaH<sub>2</sub>PO<sub>4</sub>, 5.45 g/liter KH<sub>2</sub>PO<sub>4</sub> [VWR International], pH 7) (III, IV) at 30 °C (II) or 37 °C (I, III, and IV), here referred to as the first overnight culture. A volume of 100 µl of the first overnight cultures was re-inoculated into 10 ml deoxygenated TPGY broth (I, II) or buffered TPGY broth (III) and grown anaerobically for 16 h at 30 °C (II) or 37 °C (I, III), here referred to as the second overnight culture.

All work handling viable *C. botulinum* cells was performed and all cultures were grown under anaerobic conditions in an anaerobic cabinet (MG1000 Anaerobic Work Station; Don Whitley Scientific Ltd., Shipley, United Kingdom) with an internal atmosphere of 85% N<sub>2</sub>, 10% CO<sub>2</sub>, and 5% H<sub>2</sub> (study I-IV) or in a Braun Biostat B fermenter (B. Braun, Germany) flushed with N<sub>2</sub> (study IV). All culture media were deoxygenated by boiling for 15 min (broth) or anaerobic storage for 48 h (agar plates).

#### 4.3. Minimum and maximum growth temperatures (I-III)

The GradiplateW10 temperature gradient incubator (BCDE Group, Helsinki, Finland) placed in an anaerobic workstation (MK III) was used to determine the minimum and maximum temperatures permitting growth of the 23 Group I (I) and the 24 Group II (II) strains and the maximum growth boundaries of the *hrcA* and *dnaK* mutant as well as parent *C. botulinum* strains (III). This incubator creates a uniform temperature gradient along the vertically-incubated agar plate. The temperature gradient  $g$  (in °C/mm) in the plate can be derived from the temperatures measured by two calibrated temperature sensors at a distance of 24 mm ( $T_{low}$ ) and 74 mm ( $T_{high}$ ) from the bottom edge of the agar plate using the formula  $g = (T_{high} - T_{low}) / 50 \text{ mm}$  (in °C/mm). This allows calculation of the incubation temperature  $T_{inc}$  (°C) at any point in the plate by measurement of its distance  $d$  (in mm) to  $T_{low}$  using the formula:  $T_{inc} = T_{low} + d \times g$ .

A volume of 100 µl of the second overnight culture of the strains to be studied was inoculated into 10 ml deoxygenated TPGY and grown at 37 °C (I, III) or 30 °C (II) to reach the exponential growth phase, corresponding to an OD<sub>600</sub> of 0.8 to 1.2 ODU (I, III) or 0.6 to 0.9 ODU (II). Each logarithmically-growing strain was then diluted 1:100 in

peptone water, and 25 µl of this dilution was inoculated onto anaerobic TPGY agar plates (25 g of agar per liter) using the droplet-run technique in a longitudinal direction (Korkeala *et al.*, 1990). To determine their minimum growth temperature ( $T_{\min}$ ), the strains were incubated at a temperature range from 12 °C to 18 °C for 35 d (I) or from 3 °C to 9 °C for 28 d (II). To study their maximum growth temperature ( $T_{\max}$ ), the strains were incubated at a temperature range from 40 °C to 48.5 °C (I), 33 °C to 42 °C (II), or 42 °C to 49 °C (III) for 48 h.

At the end of incubation, the growth boundary, determined as margin at which dense growth of each strain stopped, was observed optically using a stereo microscope or with the bare eye, and its distance to  $T_{\text{low}}$  measured. The  $T_{\text{inc}}$  at this growth boundary was then obtained using the above-mentioned formulas. The  $T_{\min}$  and  $T_{\max}$  of each strain were determined as the mean of  $T_{\text{inc}}$  of three (I:  $T_{\max}$ , II, III) or five (I:  $T_{\min}$ ) independent experiments.

#### 4.4. Growth characteristic experiments (I-III)

The *C. botulinum* strains were incubated in a Bioscreen C Microbiology Reader (Oy Growth Curves AB Ltd., Helsinki, Finland), that was placed in an anaerobic cabinet (MK III, Don Whitley Scientific Ltd.), to examine the growth characteristics of the 23 Group I (I), 24 Group II (II), and the mutant as well as parent strains (III). The Bioscreen C Microbiology Reader controls the incubation temperature of the bacterial culture, shakes it, and automatically measures and reports its optical density at the wavelength of 600 nm ( $OD_{600}$ ) in optical density units (ODU) in intervals.

The second overnight culture of the Group I and Group II strains (I, II) was inoculated into deoxygenated TPGY at a ratio of 1:100, mixed, and 350 µl of the inoculated culture were transferred as technical replicates into each of four wells of a 100-well microtiter plate. The Group I strains (I) were then incubated in the Bioscreen C Microbiology Reader at 20 °C for 72 h, at 37 °C for 12 h, and at 42 °C for 12 h, whereas the Group II strains (II) were incubated at 10 °C for 14 d, at 30 °C for 48 h, at 37 °C for 48 h, and at 40 °C for 48 h. The experiment was conducted in quadruplicate (I [37 °C and 42 °C]) or triplicate (I [remaining conditions], II).

The parent and the *dnaK* and *hrcA* mutant strains (III) were grown in the Bioscreen C Microbiology Reader to test growth behavior at temperature stress in buffered TPGY (pH 7) at 37 °C, 42 °C or 45 °C for 14h, to test pH stress at 37 °C in buffered TPGY adjusted to pH 5, pH 6, and pH 7.6 for up to 100 h, and to test NaCl stress at 37 °C in buffered TPGY with 3% or 3.5% (wt/vol) NaCl added for 24 h. Three biological replicates were incubated using three (pH stress) or five (temperature and NaCl stress) technical replicates.

The optical density of the *C. botulinum* culture was normalized by subtraction of the  $OD_{600}$  of inoculated medium from the measured  $OD_{600}$ . Growth curves were obtained by plotting the measured  $OD_{600}$  against the time. To calculate the maximum growth rates

(max GR) of the cultures measured in ODU per hour (ODU/h), their curves were fitted to the Baranyi and Roberts model (Baranyi & Roberts, 1994) using the DMFit 2.0 Microsoft Excel add-in program (Institute of Food Research, Norwich, UK). The differences between the mean maximum growth rates at different temperatures ( $\Delta$ GR) were calculated for each strain by subtraction of their maximum growth rates at the concerned conditions from each other (I, II).

#### **4.5. Lethal heat stress experiment (III)**

The *C. botulinum* ATCC 3502 parent and *hrcA* and *dnaK* mutant strains were anaerobically grown until reaching mid-exponential growth phase after inoculation of 100  $\mu$ l second overnight culture into 10 ml buffered TPGY broth. After sample withdrawal for enumeration the bacterial culture was sealed and exposed to a temperature above 62 °C for 2 min in a 64 °C water bath. After heat treatment, another sample for enumeration was taken. The three-tube most-probable-number approach was used for enumeration of bacterial cells to calculate the log reduction in cell number as an indicator of heat tolerance of the strains. The three strains with three biological replicates each were heat treated simultaneously.

#### **4.6. Amplified fragment length polymorphism (AFLP) analysis (II)**

The 24 Group II *C. botulinum* strains used in this study were analyzed using AFLP analysis as described by Keto-Timonen et al. (Keto-Timonen *et al.*, 2006). Briefly, preselective PCR of HindIII and HpyCH4IV (both New England Biolabs, Beverly, MA) digested and HindIII adapter and HpyCH4IV adapter (both Oligomer, Helsinki, Finland) ligated DNA samples diluted 1:2 in water was performed in a 20 ml reaction mixture using Hind-0 primer and Hpy-0 primer (both Oligomer) (72 °C for 2 min and 20 cycles of 94 °C for 20 s, 56 °C for 2 min, and 72 °C for 2 min). These templates were then 1:20 diluted in water and selective PCR amplification was conducted using labeled Hind-C primer and Hpy-A primer (both Oligomer) in a 10  $\mu$ l reaction mixture: 94 °C for 2 min; 1 cycle of 94 °C for 20 s, 66 °C for 30 s, 72 °C for 2 min; after this, the annealing temperature was lowered for 10 cycles by 1 °C each cycle to reach 56 °C, followed by additional 19 cycles at this annealing temperature of 56 °C; and a final 30-min extension at 60 °C). An ABI PRISM 310 genetic analyzer (Applied Biosystems, Foster City, CA) was used to electrophorese the denatured products of the selective PCR mixed with an internal standard on POP-4 polymer (Applied Biosystems) for 28 min at 66 °C and 15 kV.

The data were processed and analyzed and a dendrogram created using the GeneScan 3.7 fragment analysis software (Applied Biosystems) and BioNumerics software, version 4.5 (Applied Maths, Kortrijk, Belgium). The strains BL90/4, K8, K35, K51, 31-2570, 202, BL86/32, and BL86/34 had been earlier analyzed using the same

protocol and instrument (Keto-Timonen *et al.*, 2005) and were therefore included into the study without previous reanalyzing.

#### 4.7. Construction of mutants (III)

The mutant strains carrying an insertionally inactivated copy of *hrcA* (*cbo2961*) or *dnaK* (*cbo2959*) were constructed from the parental Group I *C. botulinum* strain ATCC3502 using the ClosTron gene knock out system as described by Heap *et al.* (Heap *et al.*, 2007) (Table 3). An online re-targeting algorithm (ClosTron, <http://www.clostron.com>, University of Nottingham, Nottingham, United Kingdom) was utilized to identify the target sites for the insertion of the mobile group II intron (between nucleotides 53-54 in the *hrcA* and between nucleotides 440-441 in the *dnaK* gene) and to accordingly design suitable mutagenesis plasmids (Table 3) and the primers required to construct them.

**Table 3.** Mutant, cloning, and donor strains and plasmids used in this study.

Name	Description	Source
Bacterial strains		
<i>C. botulinum</i> ATCC 3502	Parent strain	ATCC <sup>a</sup>
<i>C. botulinum</i> ATCC 3502 <i>hrcA</i> ::intron- <i>erm</i>	ClosTron insertional mutant of <i>hrcA</i> in antisense direction	III
<i>C. botulinum</i> ATCC 3502 <i>dnaK</i> ::intron- <i>erm</i>	ClosTron insertional mutant of <i>dnaK</i> in antisense direction	III
<i>E. coli</i> TOP10	Electro competent cloning strain	Invitrogen, Carlsbad, CA, USA
<i>E. coli</i> CA434	Conjugation donor strain	(Purdy <i>et al.</i> , 2002)
Plasmids		
pMTL007	ClosTron vector for mutagenesis	(Heap <i>et al.</i> , 2007)
pMTL007::CBO2961-53a	pMTL007 targeting <i>hrcA</i> in antisense direction	III
pMTL007::CBO2959-440a	pMTL007 targeting <i>dnaK</i> in antisense direction	III

<sup>a</sup>ATCC: American Type Culture Collection, Manassas, Va., USA

The mutagenesis plasmids were generated by splice overlap extension PCR according to the protocol by Heap *et al.* (Heap *et al.*, 2007) and ligation of the digested PCR product into the plasmid pMTL007. The re-targeted plasmids were cloned in electro competent *E. coli* Top10 cells (Invitrogen, Carlsbad, CA, USA), isolated, and chemically transformed into *E. coli* CA434 donor strains (Purdy *et al.*, 2002). Subsequently the plasmids were conjugated into the recipient *C. botulinum* ATCC 3502. The cells were inoculated on TPGY plates supplemented with 15 µg/ml thiamphenicol and 250 µg/ml cycloserine (both



Sigma-Aldrich) to select for *C. botulinum* cells carrying the retargeted plasmid and to inhibit growth of remaining *E. coli* cells. *C. botulinum* colonies carrying the plasmid were picked and grown in TPGY broth supplemented with 15 µg/ml thiamphenicol and integration of the mobile group II intron was induced by addition of 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG). TPGY plates containing 2.5 µg/ml erythromycin (Sigma-Aldrich) were used to select for *C. botulinum* mutants exhibiting erythromycin resistance after successful integration of the intron and activation of its erythromycin resistance gene. Intron integration at the desired target site was further confirmed by PCR using target-gene- and intron-specific primers.

#### **4.8. Heat shock experiment, batch culture (III)**

The expression of the Class I HSGs *grpE* (*cbo2960*), *dnaK* (*cbo2959*), *dnaJ* (*cbo2958*), *groES* (*cbo3299*), *groEL* (*cbo3259*), and of *hrcA* (*cbo2961*), encoding their negative regulator, were studied during vegetative growth under optimal conditions and after heat shock. A volume of 1 ml second overnight culture of the *C. botulinum* ATCC parent strain or the *hrcA* mutant strain were inoculated into 250 ml of deoxygenated buffered TPGY broth. The cultures were grown anaerobically at 37 °C until reaching mid-exponential growth, as indicated by a culture OD<sub>600</sub> of 0.9 to 1.1 ODU, and a calibrator sample of 5 ml was withdrawn. The parental strain culture to be grown as a control remained at 37 °C and further samples were taken 30 min (exponential phase of growth), 1 h 10 min (transition phase), 2 h 10 min (early stationary phase), and 5 h 10 min (stationary phase) after calibrator sample withdrawal. The parental strain and the *hrcA* mutant strain cultures to be subjected to heat shock were transferred to a water bath set to 65 °C. Immediately after reaching a culture temperature of 45 °C, the cultures were moved into an oil bath at 45 °C in an anaerobic cabinet (MG1000 Anaerobic Work Station) and a sample was taken (heat shock sample, 10 min after the calibrator sample). The cultures remained at 45 °C and further samples were withdrawn 20 min, 1 h, 2 h, and 5 h after heat shock, paralleling the sample time points of the parental strain grown as a control. The growth experiments were carried out in triplicate.

The culture samples of a volume of 5 ml each were carefully mixed with 1 ml of chilled stop solution (900 µl of 99.6% ethanol and 100 µl of phenol [Sigma-Aldrich]) to inhibit enzymatic activity, and incubated on ice for 30 min. Then they were aliquoted into 1.5 ml volumes and centrifuged for 5 min at 5000 x g at 4 °C. After supernatant removal, the cell pellets were stored at -70 °C until RNA extraction.

#### **4.9. Heat shock experiment, continuous culture (IV)**

To study the whole genome expression profile of the Group I *C. botulinum* strain ATCC 3502 exposed to heat shock and prolonged heat stress, the strain was anaerobically

grown in continuous culture in a Braun Biostat B fermenter (B. Braun) in 2 l of buffered TPGY broth at 39 °C, at a constant pH of 6.8 maintained by automatic addition of 3 M KOH (Sigma-Aldrich). The culture was initially inoculated using 10 ml first overnight culture. The culture OD<sub>600</sub> was automatically continuously measured and recorded in absorption units (AU). Feeding at a dilution rate of 0.035 h<sup>-1</sup> was initiated after an OD of 1.5 AU was reached. The *C. botulinum* culture was constantly stirred at 200 rounds per minute and flushed with N<sub>2</sub> to assure anaerobicity. Buffered TPGY for feeding was freshly autoclaved and kept anaerobic in airtight containers with N<sub>2</sub> overlay. Resazurin sodium salt (1 mg/l; Sigma-Aldrich) was used as anaerobicity indicator. The foam suppresser Antifoam A (Sigma-Aldrich) was added at a concentration of 20 mg/l to the medium.

After reaching steady-state growth, as indicated by a constant OD<sub>600</sub> of 1.6 to 1.7 AU, from about 24 h after feeding start onwards, a control sample of 5 ml was withdrawn from the bacterial culture and the incubation temperature set to 45 °C. A second sample was taken when the culture temperature reached 45 °C 8 min after temperature up-shift (defined as heat shock time point). More samples were obtained 10 min and 1 h after heat shock, during the adaptation of the culture to 45°C (18 h and 42 h after heat shock) and one last sample was taken after the culture stabilized with new steady stage continuous growth at 45°C (as indicated by a stable OD of 0.7 to 0.8 AU). A volume of 2 ml stop solution was added to the culture samples of 5 ml and gently mixed. After incubation for 30 min on ice, the samples were centrifuged at 5000 x g at 4 °C for 5 min, the supernatant removed, and the cell pellet stored at -70°C until RNA purification. The experiment was performed in duplicate, and two technical replicate samples were withdrawn at each time point.

#### **4.10. RNA isolation (III, IV)**

After lysis of the frozen cell pellet for 30 min at 37°C in 250 µl (III) or 1 ml (IV) lysis buffer (25 mg/ml lysozyme and 250 IU/ml mutanolysin [Sigma-Aldrich] in Tris-Ethylenediaminetetraacetic acid (EDTA) buffer [pH 8.0, Fluka, Biochemica, Switzerland]), total RNA was extracted using commercial spin column systems (RNeasy Mini (III) or Midi (IV) Kit, Qiagen, Hilden, Germany). Genomic DNA was removed during the isolation with an on-column DNase treatment (RNase-Free DNase Set, Qiagen), followed by a second DNase treatment after isolation using the Ambion DNA-free kit (Applied Biosystems, Life Technologies Corporation, Carlsbad, CA, USA) according to the manufacturer's instructions. The RNA concentration and quality was determined optically by measurement of the absorption units at the wavelength of 260 nm (A<sub>260</sub>) using the NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) and by electrophoresis with the 2100 Bioanalyzer using Prokaryote Total RNA Nano chips (Agilent Technologies, Santa Clara, CA, United States).

#### 4.11. Quantitative real-time reverse transcription PCR (RT-qPCR) analysis (III)

An amount of 800 ng total RNA was reverse transcribed into complementary DNA (cDNA) using the DyNAmo™ cDNA Synthesis Kit (Finnzymes, Espoo, Finland) according to the manufacturer's instructions. The reverse transcription (RT) reaction was carried out in duplicate for each RNA sample to obtain technical replicates. Minus RT controls of each RNA sample were obtained by replacement of the reverse transcriptase by RNase-free water to control for possible DNA contamination.

Quantitative real-time PCR (qPCR) reactions were performed in duplicate for each cDNA sample using DyNAmo™ Flash SYBR® Green qPCR chemistry (Finnzymes) according to the manufacturer's instructions in a Rotor Gene 3000 Real Time Thermal Cycler (Qiagen). Each reaction included 4 µl of diluted cDNA as template, target gene specific primers (Table 2 in III) in a final concentration of 0.5 µM, 10 µl 2x DyNAmo™ Flash SYBR® Green master mix, and water. The following cycling protocol was applied: polymerase activation at 95°C for 1 min, 40 cycles with 95°C for 10 sec and 60°C for 20 sec with data collection at the end of each cycle, and a final extension step for 1 min at 60°C. The primers for quantification of the Class I HSGs (*grpE*, *dnaK*, *dnaJ*, *groES*, *groEL*, and *hrcA*) and 16S *rrn* were designed using the Primer3-web 0.4.0 web-interface (<http://primer3.sourceforge.net/webif.php>) based on the published genome sequence of *C. botulinum* ATCC 3502 (Sebaihia *et al.*, 2007). Reagent contamination was controlled for by no-template controls included in each run. Primer specificity was confirmed by melt curve analysis at the end of each run.

For each primer pair, standard curves were constructed using serially-diluted pooled cDNA to calculate the amplification efficiency, and to determine suitable sample dilution and the quantification threshold for detection of fluorescence above background utilizing the Rotor Gene 3000 software version 6.1 (Qiagen). All minus RT controls underwent qPCR with melt-curve analysis using 16S *rrn* primers and none showed evidence for DNA contamination of the RNA samples.

Relative expression values of the Class I HSGs were calculated with the Pfaffl method (Pfaffl, 2001) using 16S *rrn* as the reference gene (Coesnon *et al.*, 2006; Kirk *et al.*, 2014). The mid-exponential growth phase sample was used as a calibrator to study the Class I HSG expression profile of the parent strain during normal growth at 37 °C and of the parent as well as the *hrcA* mutant strain after exposure to heat shock. The mid-exponential gene expression of the Class I HSGs of the *hrcA* mutant strain grown at 37 °C was calculated relative to that of the wild type strain as calibrator.

#### 4.12. DNA microarray analysis (IV)

To study the gene expression profile of the *C. botulinum* strain, ATCC 3502 custom-designed, *in situ*-synthesized DNA microarrays were used (8x15K; Agilent Technologies),

which covered 3,641 chromosomal (out of the total of 3,648) and all the 19 plasmid-borne CDSs of the bacterium's genome (Sebaihia *et al.*, 2007; Dahlsten *et al.*, 2014).

Of each withdrawn sample, 2 µg total RNA was reverse transcribed into cDNA and directly labeled with the fluorescent dye Cy3 or Cy5. The RT reaction mixture of 30 µl contained 5 µg of random primers, 40 U RNaseOUT™ Recombinant Ribonuclease Inhibitor, 6 µl 5x first-strand buffer, 3 µl of 100 mM DTT, 400 U SuperScript™ III Reverse Transcriptase (all Invitrogen, Life Technologies Ltd, Paisley, UK), 0.6 µl dNTP mix (25 mM dATP, 25 mM dGTP, 25 mM dTTP, 10 mM dCTP [Promega Corporation, Madison, WI, USA]), and 2 nmol Cy3-dCTP or Cy5-dCTP (GE Healthcare, Buckinghamshire, UK) and was incubated for 3 h at 46°C. After addition of 1.5 µl 20 mM EDTA and 15 µl 0.1 M NaOH, the RNA was hydrolyzed for 15 min at 70°C. The mixture was neutralized by 15 µl of 0.1 M HCl and the labeled synthesized cDNA was subsequently purified using a DNA purification column (QIAquick PCR purification kit; Qiagen) according to the manufacturer's instructions, and eluted into 44 µl elution buffer (Qiagen).

Exactly 300 ng of Cy3-labeled and Cy5-labeled cDNA samples each were mixed and 2.3 µg of salmon sperm DNA (Invitrogen) added followed by denaturation for 2 min at 95°C. The samples were cooled on ice and 10x blocking agent and 2x RPMI hybridization buffer (both Agilent technologies) were added to the cDNA mixture before loading it onto the microarray slide. After hybridization for 16 h at 65°C, the slides were washed according to the manufacturer's protocol and dried. Reference design was used by hybridization of each sample obtained after temperature up-shift against the control sample. Dye swap was performed for the technical replicates and dye bias controlled by hybridization of differently dyed control samples in one array of each microarray slide.

The microarray slides were scanned at a wavelength of 532 nm and 635 nm with a 5 µm resolution in an Axon GenePix Autoloader 4200 AL scanner (Axon Instruments Westburg, Leusden, The Netherlands). The Gene Pix Pro 6.0 software (Axon Instruments) was utilized for image processing, followed by data analysis with the R limma package (Smyth & Speed, 2003; Smyth, 2005). The foreground and local background intensities of each spot were identified by the mean and median pixel values of the spot, respectively. The "normexp" method, with an offset value of 50, was used to subtract local background from the foreground signal (Ritchie *et al.*, 2007). The signal intensities measured in the Cy5 and Cy3 channels were converted into a logarithmic (log2) scale and normalized using the loess method (Smyth & Speed, 2003).

#### **4.13. Statistical analysis (I-IV)**

The statistical program SPSS version 15.0 (SPSS Inc., Chicago, IL, USA) was used to compare the mean maximum growth rates at the different incubation conditions,  $T_{\min}$  and  $T_{\max}$  of the studied Group I and Group II *C. botulinum* strains, as well as the *hrcA* and *dnaK* mutant strains (I-III).

The differences in the expression values of Class I HSGs obtained by qPCR were analyzed using the one-sample *t* test of the above-mentioned program (III).

Statistical analysis to study the differences in expression values obtained by DNA microarrays were performed with the R limma package (Smyth, 2005) (IV). Each probe was analyzed separately using a moderated *t* test with empirical Bayes variance shrinkage (“eBayes” function). The obtained P values were translated into false discovery rate (FDR) values using a Benjamini-Hochberg adjustment. The probe with the median unmodified P value for the expression difference was chosen to represent the CDS. A CDS was considered to be differentially expressed at 39 °C and 45 °C and therefore affected by high temperature stress when a difference in expression of log<sub>2</sub>-ratio  $\geq 1$  or  $\leq -1$  and FDR  $\leq 0.05$  was detected.

CDSs differentially expressed at 39 °C and 45 °C were clustered according to their time-dependent expression pattern employing the open source software MultiExperimentViewer of the TM4 Microarray Software Suite using the k-means clustering method with Euclidean distance (Saeed *et al.*, 2003) (IV).

## 5. RESULTS

### 5.1. The strains of *C. botulinum* vary in growth at low and high temperatures

#### 5.1.1. Group I *C. botulinum* strains (I)

Substantial variation was observed with regard to temperature boundaries for growth and growth performance at different incubation temperatures for the 23 Group I *C. botulinum* strains studied (Table 4, Table 2 and 3 in I).

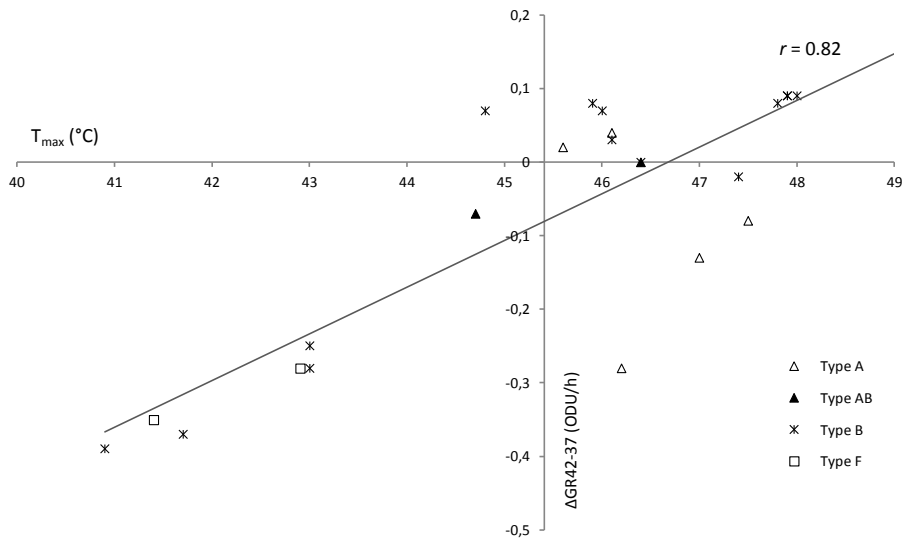
During 35 days of growth, an average  $T_{\min}$  of 14.5 °C ( $\pm 1.2$  °C) was detected considering all strains, with the lowest  $T_{\min}$  of 12.8 °C and the highest  $T_{\min}$  of 16.5 °C both recorded in type B strains (Table 4, Table 2 in I). No significant difference could be found between the different toxin types. The average  $T_{\max}$  permitting growth within 48 h was 45.4 °C ( $\pm 2.2$  °C), varying from 40.9 to 48 °C between strains (Fig 2). The highest and lowest  $T_{\max}$  were observed in type B strains. The average  $T_{\max}$  of the type F strains (42.2 °C) was significantly lower than the average  $T_{\max}$  of the other toxin types ( $P < 0.05$ ). The strains belonging to two Nordic type B clusters differed significantly in their  $T_{\min}$  as well as their  $T_{\max}$  between the two clusters ( $P < 0.05$ ). Cluster II had a lower  $T_{\min}$  and cluster I a higher  $T_{\max}$  (Table 2 in I). The strain variation within a serotype was highest for the type B strains, whereas the type A strains exhibited the widest temperature range allowing growth.

**Table 4.** Average minimum ( $T_{\min}$ ) and maximum ( $T_{\max}$ ) growth temperatures, maximum growth rates at 20 °C (max GR 20), 37 °C (max GR 37), and 42 °C (max GR 42), and differences between growth rates at 20 and 37 °C ( $\Delta$ GR20-37) and at 42 and 37 °C ( $\Delta$ GR42-37) of the studied 23 Group I *C. botulinum* strains of serotype A, AB, B, and F. Lowest and highest values obtained within the serotype in brackets.

Growth characteristic	Type A n = 5	Type AB n = 2	Type B n = 14	Type F n = 2	All n = 23
Av $T_{\min}$ (°C)	14.4 (13.3 - 15.0)	14.1 (13.8 - 15.0)	14.5 (12.8 - 16.5)	15.0 (14.4 - 15.7)	14.5 (12.8 - 16.5)
Av $T_{\max}$ (°C)	46.5 (45.6 - 47.5)	45.5 (44.7 - 46.4)	45.5 (40.9 - 48.0)	42.2 (41.4 - 42.9)	45.4 (40.9 - 48.0)
Max GR 20 (ODU/h)	0.09 (0.07 - 0.10)	0.06 (0.05 - 0.07)	0.07 (0.04 - 0.10)	0.06 (0.05 - 0.08)	0.07 (0.04 - 0.10)
Max GR 37 (ODU/h)	0.44 (0.39 - 0.51)	0.58 (0.57 - 0.59)	0.52 (0.31 - 0.62)	0.36 (0.34 - 0.37)	0.49 (0.31 - 0.62)
Max GR 42 (ODU/h)	0.35 (0.11 - 0.50)	0.54 (0.51 - 0.57)	0.47 (0.06 - 0.67)	0.04 (0.02 - 0.06)	0.41 (0.02 - 0.67)
$\Delta$ GR20-37 (ODU/h)	-0.35 (-0.43 - -0.30)	-0.52 (-0.54 - -0.50)	-0.45 (-0.52 - -0.36)	-0.29 (-0.33 - -0.26)	- 0.42 (-0.54 - -0.26)
$\Delta$ GR42-37 (ODU/h)	-0.09 (-0.28 - 0.04)	-0.03 (-0.07 - 0.0)	-0.05 (-0.39 - 0.09)	-0.32 (-0.35 - -0.28)	-0.08 (-0.39 - 0.09)

The average max GR of all studied Group I *C. botulinum* strains was 0.07 ODU/h ( $\pm 0.02$  ODU/h) at 20 °C, 0.49 ODU/h ( $\pm 0.09$  ODU/h) at 37 °C, and 0.41 ODU/h ( $\pm 0.24$  ODU/h) at 42 °C (Table 4, Table 3 in I). They varied from 0.05 to 0.10 ODU/h at 20 °C, from 0.31 to 0.62 ODU/h at 37 °C, and from 0.02 to 0.67 ODU/h at 42 °C (Table 4). The lowest max GR for type F strains was found at 42 °C, whereas the other toxin types grew on average slowest at 20 °C. At 20 °C, Nordic cluster II strains showed significantly higher max GR than cluster I strains ( $P < 0.05$ ) (Table 3 in I). Altogether eight strains had a significantly higher max GR at 42 °C than at 37 °C ( $\Delta\text{GR}_{42-37}$ ) ( $P < 0.05$ ); all of them were type B strains.

There was significant correlation between the  $T_{\text{max}}$  and the  $\Delta\text{GR}_{42-37}$  taking into account all 23 Group I *C. botulinum* strains ( $r = 0.82$ ,  $P < 0.01$ ) (Fig. 2), however, no correlation was detected considering only type A strains. No correlation between  $T_{\text{min}}$  and  $\Delta\text{GR}_{20-37}$  was observed. Of the eight type B strains with higher max GR at 42 °C compared to 37 °C, one strain had a lower than average  $T_{\text{max}}$ .



**Figure 2.** Relationship between the maximum growth temperature ( $T_{\text{max}}$ ) and the difference between maximum growth rates at 42 °C and 37 °C ( $\Delta\text{GR}_{42-37}$ ) for 23 Group I *C. botulinum* strains of serotype A (open triangles), AB (filled triangles), B (asterisks), and F (open squares). The y-axis crosses the x-axis at the average  $T_{\text{max}}$  calculated from all strains.

### 5.1.2. Group II *C. botulinum* strains (II)

As for Group I, the 24 Group II *C. botulinum* strains also demonstrated significant variation in growth characteristics at extreme temperatures (Table 5, Table 2 and 3 in II).

The average  $T_{min}$  promoting growth within 28 d of incubation was 7.3 °C ( $\pm 0.7$  °C), with the low temperature growth boundaries of the strains varying from 6.2 to 8.6 °C (Table 5, Table 2 in II). The type F strains showed, with 7.8 °C, a significantly higher average  $T_{min}$  than the type B and E strains ( $P < 0.05$ ); the largest strain variation within a toxin type was found for the type E strains. The average of all the studied 24 Group II *C. botulinum* strains'  $T_{max}$  was 38.5 °C ( $\pm 1.2$  °C); they ranged from 34.7 to 39.9 °C. Comparing the toxin types, the type E strains showed the significantly highest average  $T_{max}$  ( $P < 0.05$ ), with 39.0 °C. Type B strains had the largest variation within a toxin type and additionally exhibited the widest temperature range permitting growth on average.

Average max GRs of all studied Group II *C. botulinum* strains of 0.02 ODU/h ( $\pm 0.01$  ODU/h) at 10 °C, of 0.36 ODU/h ( $\pm 0.04$  ODU/h) at 30 °C, of 0.25 ODU/h ( $\pm 0.11$  ODU/h) at 37 °C, and of 0.06 ODU/h ( $\pm 0.05$  ODU/h) at 40 °C were obtained. However, only 11 type E strains were able to grow at a temperature as high as 40 °C in TPGY broth (Table 5, Table 3 in II). The max GRs varied from 0.01 - 0.05 ODU/h at 10 °C, from 0.29 - 0.44 ODU/h at 30 °C, from 0.08 - 0.45 ODU/h at 37 °C, and from 0.00 - 0.14 ODU/h at 40 °C (Table 5).

**Table 5.** Average minimum ( $T_{min}$ ) and maximum ( $T_{max}$ ) growth temperatures, maximum growth rates at 10 °C (max GR 10), 30 °C (max GR 30), 37 °C (max GR 37), and 40 °C (max GR 40), and differences between growth rates at 10 and 30 °C ( $\Delta GR_{10-30}$ ) and at 37 and 30 °C ( $\Delta GR_{37-30}$ ) of the studied 24 Group II *C. botulinum* strains of serotype B, F, and E. Lowest and highest values obtained within the serotype in brackets. NG: no growth observed.

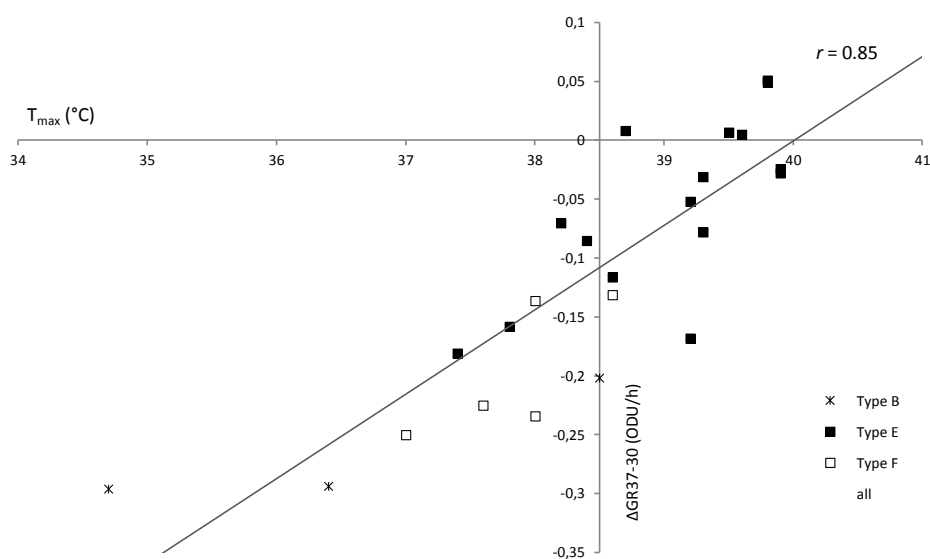
Growth characteristic	Type B n = 3	Type E n = 16	Type F n = 5	All n = 24
Av $T_{min}$ (°C)	7.0 (6.2 – 8.0)	7.1 (6.3 – 8.6)	7.8 (7.1 – 8.4)	7.3 (6.2 – 8.6)
Av $T_{max}$ (°C)	36.6 (34.7 – 38.5)	39.0 (37.4 – 39.9)	37.8 (37.0 – 38.6)	38.5 (34.7 – 39.9)
Max GR 10 (ODU/h)	0.03 (0.01 - 0.05)	0.02 (0.01 - 0.04)	0.02 (0.01 - 0.04)	0.02 (0.01 - 0.05)
Max GR 30 (ODU/h)	0.38 (0.35 - 0.40)	0.36 (0.29 - 0.44)	0.35 (0.30 - 0.41)	0.36 (0.29 - 0.44)
Max GR 37 (ODU/h)	0.11 (0.08 - 0.20)	0.30 (0.18 - 0.45)	0.16 (0.09 - 0.23)	0.25 (0.08 - 0.45)
Max GR 40 (ODU/h)	NG	0.06 (0.01 - 0.14)	NG	0.06 (0.00 - 0.14)
$\Delta GR_{10-30}$ (ODU/h)	-0.35 (-0.39 - -0.30)	-0.34 (-0.40 - -0.28)	-0.33 (-0.40 - -0.28)	-0.34 (-0.40 - -0.28)
$\Delta GR_{37-30}$ (ODU/h)	-0.26 (-0.30 - -0.20)	-0.06 (-0.18 - 0.05)	-0.20 (-0.25 - -0.13)	-0.11 (-0.25 - 0.05)



The average max GR at 37 °C of the type E strains was significantly higher than of the other toxin types ( $P < 0.05$ ). Further, five type E strains showed a higher max GR at 37 °C compared to 30 °C, however, this difference was statistically non-significant.

Taking all strains into consideration, a significant correlation between the  $T_{\max}$  and the difference between max GR at 37 and 30 °C was found ( $r = 0.85$ ,  $P < 0.05$ ) (Fig. 3). The five strains which grew faster at 37 °C than at 30 °C exhibited a higher than average  $T_{\max}$ .

Determination of the genetic background by AFLP clustering (Fig. 4 in II) divided the studied Group II *C. botulinum* strains into two type E clusters (cluster I and II) and one cluster consisting of type B and F strains (cluster III). The clusters reflected poorly in temperature-related growth behavior of the strains, nevertheless, type E cluster I strains showed a significantly higher max GR at 10 °C than cluster II strains ( $P < 0.05$ ).



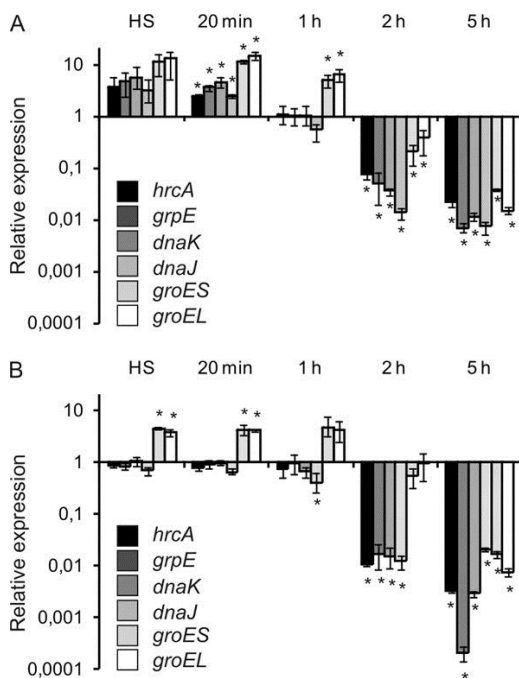
**Figure 3.** Relationship between the maximum growth temperature ( $T_{\max}$ ) and the difference between maximum growth rates at 37 °C and 30 °C ( $\Delta\text{GR}_{37-30}$ ) for 24 Group II *C. botulinum* strains of serotype B (asterisks), E (filled squares), and F (open squares). The y-axis crosses the x-axis at the average  $T_{\max}$  calculated from all strains.

## 5.2. The role of Class I HSGs in heat shock as well as pH and NaCl stress response in Group I *C. botulinum* (III)

### 5.2.1. Relative expression of Class I HSGs

During the exponential and the transition growth phase at 37 °C, the parent *C. botulinum* strain ATCC 3502 showed only marginal changes in expression of the Class I HSGs *hrcA* (*cbo2961*), *grpE* (*cbo2960*), *dnaK* (*cbo2959*), *dnaJ* (*cbo2958*), *groES* (*cbo3299*), and *groEL* (*cbo3259*), whereas significant down-regulation was observed at the later time points (Fig. 3 in III). Exposure to heat shock by temperature up-shift to 45 °C during mid-exponential growth led to an immediate 3- to 11-fold activation of all Class I HSGs (Fig. 4A). Of these, only the *groELS* operon remained 5-fold up-regulated 1 h after temperature up-shift. In the stationary phase, all studied genes were expressed at lower levels than at the earlier time points. The relative gene expression was compared to mid-exponential growth at 37 °C.

During mid-exponential growth at 37 °C, the *hrcA* mutant expressed all Class I HSGs at a more than two-fold higher level than the parent strain (Fig. 5 in III). After heat shock, activation of the *groELS* operon was observed for 1 h, whereas the *dnaK* operon remained unaffected (Fig. 4B).



**Figure 4.** Relative expression ratios of *hrcA*, *grpE*, *dnaK*, *dnaJ*, *groES*, and *groEL* at different time points after heat shock (HS) at 45 °C compared to pre-heat shock, mid-exponential growth of the *C. botulinum* ATCC 3502 wild type (A) and the *hrcA* mutant at 37 °C (B). The 16S *rrn* was used as a normalization reference. The error bars indicate the variations of three biological replicates. Relative expression ratios that differ significantly from 1 ( $P < 0.05$ ) are marked with an asterisk.

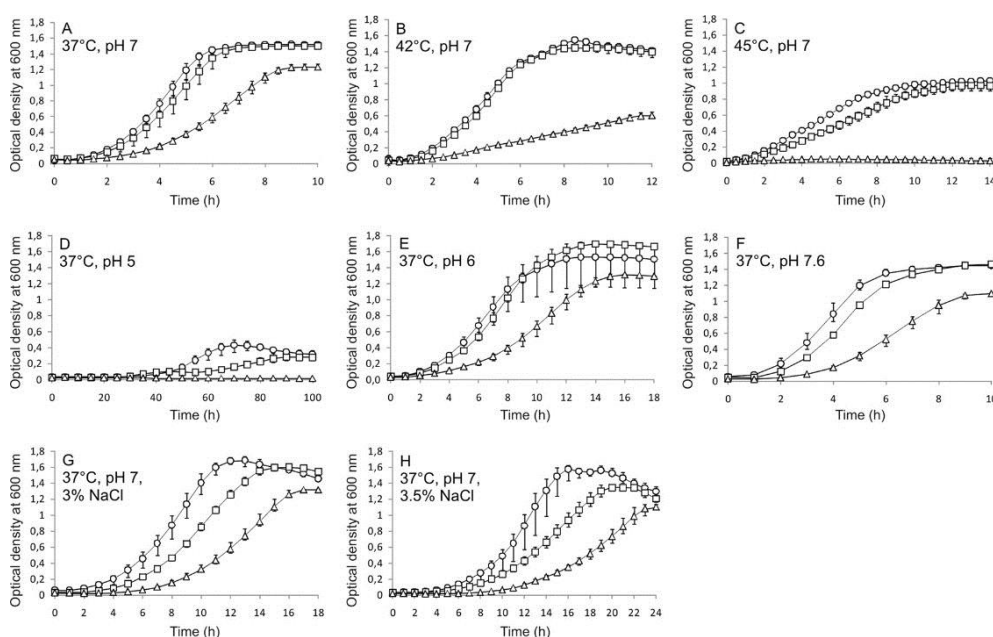
## 5.2.2. Characterization of mutant strain growth

Both mutant strains carrying the insertionally-inactivated *dnaK* or *hrcA* gene showed impaired growth and viability compared to the wild type under most tested growth conditions.

The high temperature growth boundary of both mutants was significantly reduced compared to the parent strain ( $P < 0.05$ ), with the *hrcA* mutant exhibiting a 0.9 °C and the *dnaK* mutant a 5.1 °C lowered  $T_{\max}$  (Fig. 7 in III).

Even though reduced growth of the *hrcA* mutant was observed under most tested conditions, no significant difference between its max GR during growth at 42 °C or at pH 6 could be detected compared to the parent strain (Fig. 5). The growth of the *dnaK* mutant was significantly impaired at all tested conditions ( $P < 0.05$ ).

Both mutants showed increased sensitivity to lethal heat stress. Whereas the applied heat treatment of 64 °C led to a 3.6 log reduction in cell number in the parent strain, the viable cell number of the *hrcA* mutant decreased by 5.1 log in and of the *dnaK* mutant by 8.2 log (Fig. 8 in III).



**Figure 5.** Growth curves of the *C. botulinum* ATCC 3502 wild type (open circles), *hrcA* mutant (open squares), and *dnaK* mutant (open triangles) at the indicated pH values and temperatures in buffered TPGY broth (A to F) or in TPGY broth with added NaCl (G and H). The error bars indicate the variations of three biological replicates.

### 5.3. The transcriptional response to heat shock and prolonged heat stress of Group I *C. botulinum* grown in continuous culture(IV)

Exposure of the continuously-grown *C. botulinum* to heat shock from 39 to 45 °C led to a drop in culture OD<sub>600</sub> from 1.6 to 1.7 AU before heat treatment to approximately 0.7 AU when adapted to high temperature (Fig. 1 in IV). This exposure to high temperature resulted in significant changes in the transcription of a large proportion of genes soon after heat shock, during the adaptation to high temperature and in the continuous culture adapted to 45 °C (Fig. 2 in IV).

The expression of many genes related to transcription and translation was transiently suppressed compared to growth at 39 °C, as an early response to temperature up-shift. Amongst these were genes coding RNA polymerase proteins, 30S or 50S ribosomal proteins, aminoacyl tRNA synthetases, and translation initiation as well as elongation factors (Table 1 in IV). In contrast, Class I and most Class III heat shock, as well as some SOS response related, genes were activated shortly after heat shock.

All genes coding for the proteins of the neurotoxin complex including *botA* were found to be suppressed from 10 min of growth at 45 °C onwards, being expressed at a 5- to 7-fold lower level 1 h after heat shock compared to before. They remained expressed at low level throughout the experiment, whereas no such suppression could be detected for their positive regulator *botR* (Table 1 in IV).

Prolonged high temperature stress resulted in down-regulation of the majority of sporulation-related genes in continuously-grown *C. botulinum*, including the sporulation related RNA polymerase sigma factor coding genes *sigG*, *sigE*, *sigF*, and *sigK*. Many of these genes were suppressed from as early as 1 h after heat shock onwards, whereas down-regulation of their master regulator *Spo0A* coding gene was observed only in the heat-adapted culture. In contrast, increased transcription of the majority of chemotaxis and motility-related genes was detected as a response to long-term exposure to high incubation temperature. However, some of these genes were transiently suppressed 1 h after heat shock, amongst these were the flagellar-specific polymerase sigma factor coding gene *sigD* and several genes coding for flagellin, the main structural component of the bacterial flagella (Table 1 in IV).

Both major loci of genes assigned to be related to phage and IS elements were affected by temperature stress. The first locus was up-regulated predominantly during the adaption and in the heat-adapted culture. The second locus was expressed differently; a large part of it was activated by heat already 1 h after heat shock, whereas the remaining part was suppressed at high temperature. Amongst the up-regulated genes of the second locus were a RNA polymerase sigma factor coding gene and other genes related to transcriptional regulation.

Genes related to the acetone-butanol-ethanol fermentation pathway were also affected by high temperature stress. The fermentation pathway to convert acetyl-CoA to butyryl-CoA, the basic compound for butanol and butyrate production, was strongly down-

regulated during heat stress, whereas the gene coding for aldehyde-alcohol dehydrogenase was up-regulated. In addition, a number of genes related to the metabolism and transport of the carbohydrates glycerol, sorbitol, and trehalose were activated by heat (Table 1 in IV).

The *C. botulinum* cells responded to heat stress with reduced transcription of genes coding secreted proteases throughout the experiment. Further, a suppression of genes of the leucine and phenylalanine metabolism pathways upon heat shock was found. Of these, the leucine-related genes remained suppressed, whereas the phenylalanine-related genes were activated at later time points. Genes for the proline reductase complex were also activated at the late time points, whereas glycine reductase complex genes were transiently suppressed after heat shock. Further, a number of genes related to the biosynthesis of the sulfur-containing amino acids, cysteine and methionine, were found to be induced by heat.

## 6. DISCUSSION

### 6.1. Variation between strains of *C. botulinum* regarding growth at low and high temperatures (I, II)

Unexpectedly large strain variation was found with regard to temperature boundaries for growth and growth performance at different temperatures amongst the 23 Group I and the 24 Group II *C. botulinum* strains included in the studies.

The  $T_{\min}$  and  $T_{\max}$  variation within Group I strains (12.8 to 16.5 °C and 40.9 to 48 °C, respectively) was higher than within Group II strains (6.2 to 8.6 °C and 34.7 to 39.9 °C, respectively). This finding was interesting in light of the genetic background the strains reflected: when studied by AFLP analysis, Group I strains clustered more closely together than Group II strains, indicating their lower genetic diversity (Keto-Timonen *et al.*, 2005; Keto-Timonen *et al.*, 2006). This knowledge could lead to the assumption that the Group I strains, being more closely related, would also be physiologically more similar compared to Group II strains. This assumption could not be supported in terms of growth-limiting temperatures for the studied strains.

In general, the genetic background of the Group I and the Group II strains included in the studies failed to reflect well in their growth behavior at stressful temperature. This finding has been recently confirmed for Group II *C. botulinum* with regard to  $T_{\min}$  (Stringer *et al.*, 2013). In Group I and Group II, relatively closely genetically-related strains possessing significantly different growth traits were found. Nevertheless, Group I *C. botulinum* strains representing the Nordic type B cluster I or II (Nevas *et al.*, 2005; Lindström *et al.*, 2009) showed very similar growth limits within, and significantly different limits between, the genetic clusters, with cluster II strains showing more psychrotrophic tendencies with lower  $T_{\min}$ s and higher max GRs at 20 °C. These two clusters have also been shown to differ in their cadmium and arsenic resistance (Lindström *et al.*, 2009).

In both Group I and Group II, strains were found that grew faster at temperatures higher than at their expected optimum growth temperatures (Lindström & Korkeala, 2006). Eight Group I type B strains grew significantly faster at 42 than at 37 °C and five Group II type E strains grew faster at 37 than at 30 °C. Most of these strains also exhibited a  $T_{\max}$  above the average of all strains and thus appeared to tolerate high temperature better than other strains. These observations indicate that the optimal growth temperature for many Group I and Group II *C. botulinum* strains may actually be noticeably higher than commonly expected. Interestingly, one Group I type B strain growing faster at the higher temperature had a  $T_{\max}$  lower than average. In contrast, three type A strains with high  $T_{\max}$  grew faster at 37 than at 42 °C and also the type E strain with the highest  $T_{\max}$  grew faster at 30 than at 37 °C. These findings emphasize that, even though good correlation between growth characteristics at high temperature was found, on an individual

strain level, prediction from one growth trait to another may not be possible and lead to underestimation of risks.

Within Group II, the type E toxin-producing strains appeared to tolerate high temperature better than type B or F strains. The only strains able to grow at 40 °C in broth were type E strains, they showed the highest average GR at 37 °C, and five of them grew faster at 37 than at 30 °C, the commonly-quoted optimal growth temperature for Group II *C. botulinum* (Lindström & Korkeala, 2006; Carter & Peck, 2015). Therefore the type E strains seemed to show a more mesophilic growth tendency.

Even though *C. botulinum* type E strains have a high prevalence in fish and the fish-production environment (Huss, 1980; Hielm *et al.*, 1998a; Hyytiä *et al.*, 1998), they account for only a relatively small number of botulism outbreaks related to commercial food (Korkeala *et al.*, 1998; King *et al.*, 2009). The results indicate that commonly-recommended storage temperatures and times may be sufficient to prevent type E toxin formation of most strains in fishery products. Nevertheless, the studied Group II strains do pose a special risk in REPFEDs. For all of them, a  $T_{min}$  permitting growth below 9 °C, a temperature frequently found in household refrigerators (James *et al.*, 2008), was found.

Larger variation in the studied growth characteristics at high temperature than at low temperature was detected. This is in accordance with a study examining the probability of growth of *C. botulinum*, which found a larger variation amongst Group I strains at 47 °C than at 12 °C (Jensen *et al.*, 1987). Larger strain variation at high temperature has also been described for other foodborne pathogens like *E. coli* and *B. cereus* (Membré *et al.*, 2005). The temperature dependence of protein structure and thus temperature sensitivity of enzymes is a possible explanation for this finding (Dobson, 2003; Richter *et al.*, 2010). At high temperature, conformational changes in protein structure can hamper their function, which in turn might inhibit bacterial growth. Differences in protein and enzyme composition of individual *C. botulinum* strains and possibly even small alterations in amino acid sequences could therefore lead to differences in temperature sensitivity and in growth characteristics between strains, especially at stressful high temperature.

Distinct strain variation was observed with regard to growth at stressful temperatures for *C. botulinum* strains. Furthermore, the possibility of higher-than-assumed optimal growth temperatures, as well as the difficulty to predict the studied growth traits for individual strains was shown. These findings must be carefully taken into consideration when applying risk-assessment methods and designing challenge studies to evaluate the safety of food products with regard to *C. botulinum* (Wachnicka, 2014).

## **6.2. Importance of Class I HSGs in stress response in Group I *C. botulinum* (III)**

After exposure of batch-culture grown *C. botulinum* ATCC 3502 to heat shock from 37 to 45 °C, transiently-induced expression of the two Class I HSG operons *dnaK* and

*groELS* was observed. Class I HSGs code for molecular chaperones (Sebahia *et al.*, 2007), which assist protein folding and are negatively regulated by HrcA in *B. subtilis* (Schulz & Schumann, 1996). Activation of these genes has recently been reported for *C. botulinum* 15 min after heat shock using a genome-wide gene expression profile approach with DNA microarrays (Liang *et al.*, 2013) and is in agreement with the earlier reported increase of DnaJ protein expression in *C. botulinum* after heat shock (Shukla & Singh, 1999). Activation of Class I HSGs for 20 min (*dnaK* operon) up to 1 h (*groELS* operon) after heat shock was detected here (Study III), which was considerably longer than reported for *B. subtilis* (Schmidt *et al.*, 1992; Wetzstein *et al.*, 1992). Similar observations have been made for *C. acetobutylicum* (Narberhaus & Bahl, 1992; Narberhaus *et al.*, 1992). This indicates that Class I HSG encoded molecular chaperones may play a more important role in the heat stress response of clostridia than in other Gram-positive bacteria, which could be explained by their need to compensate the lack of homologs of SigB regulated Class II HSGs of the *B. subtilis* (Hecker *et al.*, 2007).

The insertional inactivation of *hrcA* in Study III resulted in over-expression of Class I HSGs during mid-exponential growth similar to *B. subtilis* (Schulz & Schumann, 1996) and therefore confirmed its predicted function as negative regulator of Class I HSGs in *C. botulinum* (Sebahia *et al.*, 2007). Interestingly, heat shock led to further activation of the *groELS* operon in the mutant, whereas the *dnaK* remained unaffected. This observation and the fact that heat shock led to a longer activation of the *groELS* than the *dnaK* operon in the wild type strain indicate the presence of a further, currently-unknown regulatory pathway for the *groELS* operon. However, no consensus site for SigK, which was identified in a type D strain (Sagane *et al.*, 2003), could be detected upstream of the *groELS* operon in *C. botulinum* ATCC 3502.

The mutant strains carrying the insertionally inactivated *hrcA* or *dnaK* gene were viable under most tested conditions, however showed limitations in their growth performance, reduced recovery after lethal heat stress, and a lower  $T_{max}$  compared to the parental strain. The *dnaK* mutant was more strongly restricted in its growth than the *hrcA* mutant under all tested conditions. This highlights the importance of functional *dnaK* for *C. botulinum* for growth under stressful, but also under optimal growth conditions, similar to *E. coli* and *B. subtilis* (Paek & Walker, 1987; Schulz *et al.*, 1995). The molecular chaperone DnaK is involved in correct folding of nascent proteins during normal cell growth and in repair and stabilization of denatured proteins (Schröder *et al.*, 1993; Hartl & Hayer-Hartl, 2002) and therefore plays a major role in bacterial survival, especially under heat stress resulting in protein denaturation.

Under optimal growth conditions (pH 7.0 at 37 °C) the *hrcA* mutant strain showed a gently-reduced maximum GR. But interestingly, during growth under mild stress conditions (42 °C or pH 6.0), no significant difference was detected compared to the parental strain. This observation could be explained by a possible positive effect of continuously over-expressed Class I HSGs, leading to elevated levels of molecular chaperons in the bacterial cell. These might promote bacterial growth under mild stress



conditions. However, when exposed to more severe environmental stress, the inability of the *hrcA* mutant to further activate the *dnaK* operon resulted in impaired growth.

Both mutant strains showed restricted growth under increasing acidic and osmotic stress. The involvement of Class I HSGs in the response of *C. botulinum* to environmental stresses other than heat resulting in intracellular accumulation of denatured and aggregated protein seems likely. Similarly, the cold shock genes of *C. botulinum* have been observed to respond to other stresses (Derman *et al.*, 2015). Exposure to high temperature of *B. subtilis* and a number of foodborne pathogens has been shown to be able to lead to cross-protection against other stresses (Volker *et al.*, 1992; Wesche *et al.*, 2009). This is an important factor when considering the role of *C. botulinum* as a foodborne pathogen. Exposure to mild, sublethal stresses during food processing might result in activation of the molecular chaperone system and subsequently protect *C. botulinum* from other stresses, especially when applying hurdle technology as a food safety measure.

### **6.3. Modulation of gene expression patterns of Group I *C. botulinum* grown in continuous culture after heat shock and during adaptation to high temperature (IV)**

The transcriptomic analysis of continuously-grown *C. botulinum* ATCC 3502 exposed to heat shock at 45 °C showed transient suppression of transcription- and translation-related genes within 1 h, indicating a temporary growth arrest of the culture. Liang *et al.* (2013) made a similar observation at the transcriptional level by 15 min after heat shock of mid-exponential *C. botulinum* batch culture, but this change was not detected by OD measurements in study III. The activation of Class I HSGs, which have been related to heat shock response in *C. botulinum* earlier (study III), Class III HSGs, and SOS response genes further proved induction of a heat shock response in this experimental set up.

Down-regulation of *botA* and the ANTP coding genes was observed from 10 min after heat shock, persisting throughout the experiment. BoNT expression therefore seemed to be suppressed at the transcriptional level in heat-stressed *C. botulinum*. This finding is in agreement with an early study detecting reduced toxin levels in *C. botulinum* culture grown in a fermenter at 45 °C (Siegel & Metzger, 1979), but contrasts reports that no differences in BoNT levels or *botA* transcription levels were identified comparing batch culture grown *C. botulinum* at 37 and 44 °C (Couesnon *et al.*, 2006). However, it seems reasonable that cellular functions ensuring survival might be prioritized over dispensable processes with high energy costs like large toxin complex production when bacteria are exposed to severe stress. The impact of growth phase and environmental influences (like lack of nutrients, changes of pH, or other factors involved in toxin production by *C. botulinum* when grown in batch culture) have been minimized by the experimental set up, which could serve as an explanation for this apparent discrepancy. Interestingly, *botR*, the positive regulator of toxin expression (Marvaud *et al.*, 1998), showed mild activation, underlining the presence of a *botR*-independent active negative regulatory network

controlling *botA* expression, most likely in response to environmental stimuli. Unfortunately, there was insufficient data for the only identified negative regulator of toxin expression (Zhang *et al.*, 2013). However, the transcription of several genes for TCS and transcriptional regulators was affected by heat stress, which might serve as a basis for future studies.

Sporulation is an efficient measure that bacteria employ to withstand adverse environmental conditions, since spores are extremely resistant to heat, desiccation, UV-light, and radiation (Stephens, 1998). It has been linked to toxin production in some pathogenic clostridia (Li & McClane, 2010; Saujet *et al.*, 2011; Al-Hinai *et al.*, 2015). Surprisingly, suppression of the majority of sporulation genes, including sporulation-related RNA polymerase sigma factor coding genes, was observed especially during adaptation and in the heat-adapted continuous *C. botulinum* ATCC 3502 culture. Apparently, sporulation is not utilized in the heat stress response of *C. botulinum*. Reduced expression of sporulation-related genes has also been observed in *B. subtilis* exposed to secretion stress (Lulko *et al.*, 2007). One reason might be that spores formed under heat stress most likely contain high levels of damaged proteins and DNA. They might therefore be dysfunctional, unable to germinate or outgrow into culture. Further, heat stressed *C. botulinum* cells might not be able to form spores due to impaired proteins in the spore formation apparatus or lack of metabolic resources. Sporulation might thus be an inappropriate survival mechanism if spores are produced under severe heat stress.

Chemotaxis- and motility-related genes were activated during the adaptation and in the continuous *C. botulinum* culture adapted to heat. Increase in motility to possibly occupy more favorable environmental niches can serve as an alternative survival strategy in bacteria (Stephens, 1998). Sporulation and motility, both growth-phase dependent during batch culture growth of bacteria, appeared to be oppositely regulated in response to heat stress in *C. botulinum*. This regulation pattern has been previously described in different bacteria (Aizawa *et al.*, 2002; Tomas *et al.*, 2003; Lulko *et al.*, 2007) and has been related in *B. subtilis* to lack of competition for SigD, the motility-related sigma factor, due to reduced expression of sporulation-related sigma factors (Lulko *et al.*, 2007). In contrast, the non-spore-forming *L. monocytogenes* and *E. coli* exhibit a non-motile phenotype at high temperature (Griffin & Robbins, 1944; Li *et al.*, 1993). Interestingly, 1 h after heat-shock reduced transcription was detected for *sigD*, encoding the above-mentioned sigma factor, and several flagellin coding genes. Their activation at later time points might suggest that increase in motility and flagella serves as a longer term mechanism to adapt to high temperature rather than as an acute heat shock response. Since flagella synthesis is a highly energy-consuming process, this observation seems biologically reasonable.

A large proportion of the genes on the two *C. botulinum* ATCC 3502 prophages were activated by heat, most strongly during the adaptation and in the heat-adapted culture, but many of them already soon after heat shock. Heat induction of the lytic cycle as reported for *E. coli* and *B. subtilis* (Lieb, 1964; Armentrout & Rutberg, 1971) seems unlikely, however, since most structural prophage genes were not induced. Interestingly, a number of regulatory genes encoded from the prophages, including an RNA polymerase sigma

factor coding gene, were activated. Lysogenic phages, which are integrated into the host genome can contribute to fitness and pathogenicity of the bacterial host by transfer of beneficial genes (Brussow *et al.*, 2004; Fortier & Sekulovic, 2013). Interestingly, a prophage-encoded sigma factor has been related to increased viability and biofilm formation in *B. anthracis* (Schuch & Fischetti, 2009). The prophages of *C. botulinum* ATCC 3502 appear to be unique for this strain (Sebaihia *et al.*, 2007). Therefore unique prophage-encoded regulatory genes might affect the phenotype of this particular strain and even account for its relatively high temperature tolerance as shown by the high  $T_{\max}$  compared to other Group I strains (I).

Many genes related to energy metabolism of *C. botulinum* showed changes in their expression after heat shock. Genes of the acetone-butanol-ethanol fermentation pathway, that have recently been shown to play a role in the cold tolerance of *C. botulinum* (Dahlsten *et al.*, 2014), were strongly suppressed from 1 h after heat shock onwards, most likely leading to reduced butanol and butyrate levels in the culture. Butyrate has been reported to induce toxin synthesis in *C. difficile* (Karlsson *et al.*, 2000). Lowering butyrate levels in the culture during heat stress might therefore have an impact on toxin production in *C. botulinum* as well. The proteolytic activity of the culture appeared reduced as secreted protease coding genes were suppressed soon after heat shock and remained down-regulated. Further, changes in expression levels of genes related to fermentation of amino acids by the Stickland reaction (Stickland, 1934; Sebaihia *et al.*, 2007), genes related to the metabolism of sulfur containing amino acids, and the corresponding amino acid transporters were observed in response to heat stress. All these findings indicate dramatic changes in the energetic status of the *C. botulinum* cell when exposed to heat stress. Interestingly, a link between BoNT production and cell metabolism has been recently made as CodY, coding a GTP-sensing global regulator, has been identified as a positive regulator of *botA* expression (Zhang *et al.*, 2014). However, deeper understanding of the connection between BoNT production and energy metabolism in *C. botulinum* requires further investigation.

## 7. CONCLUSIONS

1. Group I and Group II *C. botulinum* strains varied significantly within their groups with relation to growth-limiting temperatures and their capability to grow at extreme temperatures. This strain variation was particularly large at high temperature. In both groups, strains with considerably high  $T_{\max}$  were detected. Most of these strains also grew faster at high incubation temperature and are therefore expected to have optimal growth temperatures clearly above the commonly quoted optimum. Even though good correlation between  $T_{\max}$  and growth rates at high temperature was detected, a number of strains for which this did not apply were found. Therefore prediction from one growth trait to another appears to be difficult, especially at high temperature. These observations are of significance with regard to the development of risk assessment methods and the design of challenge studies to estimate the risk of *C. botulinum* growth and BoNT production in food.
2. The Group I *C. botulinum* strain ATCC 3502 employed Class I HSGs in the acute response to heat stress. The annotated role of HrcA as the negative regulator of these genes has been confirmed as a mutant strain harboring an insertionally-inactivated *hrcA* gene significantly over-expressed Class I HSGs. The importance of the molecular chaperone DnaK in the growth of *C. botulinum* in optimal and stressful conditions has been demonstrated, as a mutant carrying an insertionally-inactivated *dnaK* gene showed impaired growth characteristics in all tested conditions. Both mutant strains were growth restricted, not only under heat stress, but also under acidic and saline stress. This finding indicates that heat activation of Class I HSGs could lead to subsequent cross-protection against other stresses, which might be of relevance in terms of food safety when hurdle technology is applied to prevent growth of *C. botulinum*.
3. During acute heat stress, continuously grown *C. botulinum* ATCC 3502 utilized genes of the SOS response system in addition to Class I and Class III HSGs during heat shock response. High temperature led to reduced expression of *botA*, as well as ANTP coding genes under the tested conditions. No initiation of production of dormant spores as a survival strategy against heat stress was observed in *C. botulinum*, whereas lasting activation of motility-related genes indicated an attempt of the culture to migrate towards a more beneficial growth environment. Many prophage genes were over-expressed under heat stress; these unique prophages might therefore contribute to relatively high heat tolerance of the strain. Intensive metabolic remodeling was associated with the heat stress response, especially in the long-term adaptation to heat in continuously-grown *C. botulinum*, and might influence the culture's ability to form BoNT.

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