Applied and Environmental Microbiology, May 2011, p. 2823–2830 Vol. 77, No. 9
0099-2240/11/$12.00 doi:10.1128/AEM.02633-10

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[▽]

Katja Selby,[1]* Miia Lindström,[1] Panu Somervuo,[1] John T. Heap,[2] Nigel P. Minton,[2] and Hannu Korkeala[1]

Department of Food Hygiene and Environmental Health, Faculty of Veterinary Medicine, University of Helsinki, Helsinki, Finland,[1] *and Center for Biomolecular Sciences, University of Nottingham, Nottingham, United Kingdom*[2]

Received 11 November 2010/Accepted 20 February 2011

Class I heat shock genes (HSGs) code for molecular chaperones which play a major role in the bacterial response to sudden increases of environmental temperature by assisting protein folding. Quantitative reverse transcriptase real-time PCR gene expression analysis of the food-borne pathogen *Clostridium botulinum* grown at 37°C showed that the class I HSGs *grpE*, *dnaK*, *dnaJ*, *groEL*, and *groES* and their repressor, *hrcA*, were expressed at constant levels in the exponential and transitional growth phases, whereas strong downregulation of all six genes was observed during stationary phase. After heat shock from 37 to 45°C, all HSGs were transiently upregulated. A mutant with insertionally inactivated *hrcA* expressed higher levels of class I HSGs during exponential growth than the wild type, followed by upregulation of only *groES* and *groES* after heat shock. Inactivation of *hrcA* or of *dnaK* encoding a major chaperone resulted in lower maximum growth temperatures than for the wild type and reduced growth rates under optimal conditions compared to the wild type. The *dnaK* mutant showed growth inhibition under all tested temperature, pH, and NaCl stress conditions. In contrast, the growth of an *hrcA* mutant was unaffected by mild temperature or acid stress compared to the wild-type strain, indicating that induced class I HSGs support growth under moderately nonoptimal conditions. We show that the expression of class I HSGs plays a major role for survival and growth of *C. botulinum* under the stressful environmental conditions that may be encountered during food processing or growth in food products, in the mammalian intestine, or in wounds.

The exposure of bacteria to a sudden increase in temperature leads to the heat shock response, in which there is an increased synthesis of heat shock proteins (HSPs) (12), encoded by heat shock genes (HSGs). In the Gram-positive model organism *Bacillus subtilis* these HSPs are, depending on their type of transcriptional regulation, assigned to different classes, of which the class I HSPs are the most widespread among eubacteria (20). Class I HSGs are organized in two operons (*dnaK* and *groE* operon). In *B. subtilis* they are negatively regulated by HrcA, encoded by the first gene of the *dnaK* operon (18). HrcA interacts with a perfect inverted repeat of 9 bp separated by a 9-bp spacer, the CIRCE element, which precedes both operons (18). The expression of class I HSGs is induced by the presence of denatured proteins in the cytosol (9). Class I HSPs are of importance not only during heat shock but also for growth under nonstressed conditions, and they play a further role in the bacterial response to environmental stresses other than heat that cause protein denaturation (8, 9, 25). They act as molecular chaperones in the cytosol and prevent protein misfolding and aggregation by stabilization of unfolded or partially folded proteins (3). The major chaperone, DnaK, a nonribosome-binding protein in the bacterial cytosol, is a central player in the heat shock response. Together with the auxiliary proteins DnaJ and GrpE, DnaK binds and releases newly synthesized polypeptide chains in an ATP-dependent manner. It has been suggested that DnaK also prevents intramolecular misfolding and thus assists in posttranslational folding of multidomain proteins (3).

The Gram-positive, spore-forming, anaerobic bacterium *Clostridium botulinum* produces the potent botulinum neurotoxin, the causative agent of botulism in humans and animals. Food-borne botulism is the result of intoxication with botulinum neurotoxin that occurs due to the intake of preformed toxin with food after growth of toxin-forming culture in the product. In cases of infant, intestinal, and wound botulism, the disease is caused by toxin production *in vivo* after infection and growth of toxigenic *C. botulinum* (4). During growth in food, in the mammalian intestinal tract, or in wounds, *C. botulinum* is subjected to numerous different environmental challenges. These include rapid heating and cooling, salt and other preservatives in food products, changes in pH, bile salts and proteases in the intestinal tract, and acidic pH and the host immune response in inflamed tissues. To survive these environmental stresses, *C. botulinum* needs effective defense mechanisms. Although it has been shown that *C. botulinum* alters its protein synthesis after heat shock (22) and that molecular chaperones most likely play a major role in the stress response of *C. botulinum*, little information about class I HSPs in this organism is available. To date, the chaperone DnaJ has been identified in a *C. botulinum* type A strain (23), and the chaperone GroEL and its cochaperone GroES have been characterized in a *C. botulinum* type D strain (16). Besides the genes

* Corresponding author. Mailing address: Department of Food Hygiene and Environmental Health, Agnes Sjöberginkatu 2, 00014 Helsinki University, Finland. Phone: 358 9 19157199. Fax: 358 9 19157101. E-mail: katja.selby@helsinki.fi.

▽ Published ahead of print on 4 March 2011.

TABLE 1. Strains and plasmids used in the study

Strain or plasmid	Description	Reference or source
Strains		
C. botulinum ATCC 3502	Wild-type strain	ATCC, Manassas, VA
C. botulinum ATCC 3502 *hrcA*::Ll.*ltrB-erm*	Strain with insertional inactivation of the *hrcA* gene	This study
C. botulinum ATCC 3502 *dnaK*::Ll.*ltrB-erm*	Strain with insertional inactivation of the *dnaK* gene	This study
E. coli TOP10	Electro-competent cloning strain	Invitrogen, Carlsbad, CA
E. coli CA434	Conjugation donor strain	15
Plasmids		
pMTL007	ClosTron mutagenesis vector	5
pMTL007::*hrcA*-53a	ClosTron mutagenesis vector, intron retargeted to *hrcA*	This study
pMTL007::*dnaK*-440a	ClosTron mutagenesis vector, intron retargeted to *dnaK*	This study

coding for DnaJ, GroEL, and GroES, the published genome sequence of *C. botulinum* strain ATCC 3502 suggests the presence of genes coding for GrpE, a heat shock protein, HrcA, the negative regulator of class I HSGs, and DnaK, a major molecular chaperone (21). These genes are organized in the *dnaK* and the *groE* operons (21).

Here, we show that genes coding for members of the class I HSPs in *C. botulinum* were strongly induced after heat shock. Two genes of major importance in the heat stress response in *B. subtilis* (18, 19, 20) were selected for mutational analysis. These included *hrcA* (CBO2961), predicted to encode the negative regulator of the *dnaK* and *groE* operons, and *dnaK* (CBO2959), predicted to encode a major molecular chaperone, DnaK, that is important for protein folding (3). Increased expression of class I HSGs in an *hrcA* mutant confirmed that HrcA negatively regulates class I HSGs in *C. botulinum*. Inactivation of *hrcA* or *dnaK* demonstrated that the two genes play a significant role in the growth of *C. botulinum* under optimum conditions, but especially in adverse environments, such as high temperature, pH stress, and high NaCl concentration.

MATERIALS AND METHODS

Strains, plasmids, and growth conditions. The bacterial strains and plasmids used are listed in Table 1. The *C. botulinum* ATCC 3502 parent strain was stored as a spore stock culture at 4°C; the mutant strains were stored as frozen stock cultures at −70°C in 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]) supplemented with 20% glycerol. Routinely, the *C. botulinum* strains were grown in deoxygenated TPGY broth or plated on deoxygenated TPGY plates (10 g/liter agar) at 37°C 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_2, 10% CO_2, and 5% H_2. When required, the medium was supplemented with 2.5 μg/ml erythromycin, 250 μg/ml cycloserine, or 15 μg/ml thiamphenicol (all antibiotics from Sigma-Aldrich, Steinheim, Germany). *Escherichia coli* was grown in Luria-Bertani (LB) medium (Difco) or on 2× TY agar plates (16 g/liter tryptone, 10 g/liter yeast extract, 5 g/liter NaCl [J. T. Baker, Deventer, Netherlands]) at 37°C under aerobic conditions. When required, the medium was supplemented with 25 μg/ml chloramphenicol.

Construction of the *hrcA* and *dnaK* knockout mutants. Mutants were constructed by directed insertional inactivation of *hrcA* (CBO2961) (*C. botulinum* ATCC 3502 *hrcA*::Ll.*ltrB-erm*; here, the *hrcA* mutant) and *dnaK* (CBO2959) (*C. botulinum* ATCC 3502 *dnaK*::Ll.*ltrB-erm*; here, the *dnaK* mutant) of the wild-type strain ATCC 3502 using the ClosTron gene knockout system as described by Heap et al. (5), based on the mobile group II intron from *ltrB* of *Lactococcus lactis* (Ll.*ltrB*). Target sites for the insertions were identified, and mutagenesis plasmids were designed using an online tool (ClosTron, N. Minton, J. Heap, et al., University of Nottingham, Nottingham, United Kingdom). For the insertion into *hrcA*, the plasmid pMTL007::*hrcA*-53a was constructed by splicing by overlap extension PCR using the primers *hrcA*-IBS, *hrcA*-EBS1d, *hrcA*-EBS2, and EBS universal primer (Table 2) (all oligonucleotides were obtained from Oligomer, Helsinki, Finland), followed by ligation of the HindIII/BsrGI-digested PCR product into the plasmid pMTL007. For the insertion into *dnaK*, the plasmid pMTL007::*dnaK*-440a was constructed in the same way using the primers *dnaK*-IBS, *dnaK*-EBS1d, *dnaK*-EBS2, and EBS universal primer (Table 2). The plasmids were transformed into electro-competent *E. coli* TOP10 cells by electroporation. For verification of correct retargeting, the purified plasmids pMTL007::*hrcA*-53a and pMTL007::*dnaK*-440a were sequenced using the primer 5402F-F1 (Table 2). Plasmids with the intended sequence were chemically transformed into the *E. coli* CA434 donor and conjugated into the recipient *C. botulinum* strain ATCC 3502 (5). The cells were restreaked on plates supplemented with cycloserine and thiamphenicol to select for *C. botulinum* cells carrying the retargeted plasmid. Cycloserine- and thiamphenicol-resistant *C. botulinum* cells were grown in broth supplemented with thiamphenicol until stationary phase, and integration of the group II intron into the genome was induced with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG). Successful integration resulted in activation of the erythromycin resistance gene of the intron, thus leading to erythromycin-resistant *C. botulinum* mutants. The correct position of the intron in the *C. botulinum* genome was verified by PCR using the primers upstream and downstream of the insertion site, the flanking primers *hrcA*-53-forw and *hrcA*-53-rev (Table 2) for the *hrcA* mutant strain and *dnaK*-440-forw and *dnaK*-440-rev (Table 2) for the *dnaK* mutant strain, to amplify the region containing the insertion and by a second PCR using the insert-specific EBS universal primer and the flanking primer *hrcA*-53-rev for the *hrcA* mutant strain or *dnaK*-440-rev for the *dnaK* mutant strain.

Growth experiments for relative gene expression analysis. To observe the expression of the class I heat shock chaperone genes *grpE*, *dnaK*, *dnaJ*, *groES*, and *groEL* and for *hrcA*, encoding their negative regulator (Fig. 1), in the *C. botulinum* strain ATCC 3502 during growth at 37°C without heat shock, the strain was grown anaerobically in 10 ml of deoxygenated buffered TPGY broth (6.25 g/liter NaH_2PO_4, 5.45 g/liter KH_2PO_4 [VWR International]) (pH 7) at 37°C for 24 h. A volume of 100 μl of the culture was reinoculated into 10 ml of fresh buffered TPGY broth and incubated at 37°C for 16 h overnight (here referred to as the second overnight culture). A volume of 1 ml of the second overnight culture was used to inoculate 250 ml of buffered TPGY. The culture was grown anaerobically at 37°C until it reached the mid-exponential growth phase corresponding to an optical density at 600 nm (OD_{600}) of 0.9 to 1.1 units (calibrator sample, or time zero [t_0]) (Fig. 2), when the first sample was taken. Further samples were withdrawn at 30 min (exponential), 1 h 10 min (late-exponential phase), 2 h 10 min (transition phase), and 5 h 10 min (stationary growth phase) after time zero (Fig. 2). Five-milliliter samples were transferred into 15-ml Falcon tubes containing 1 ml of chilled stop solution (900 μl of 99.6% ethanol and 100 μl of phenol [Sigma-Aldrich]), gently mixed, and incubated on ice for 30 min. The samples were divided into 1.5-ml aliquots and centrifuged for 5 min at 5,000 × *g* at 4°C. The supernatant was removed, and the cell pellets were stored at −70°C until RNA purification. The experiment was performed in triplicate.

To study the expression of *hrcA*, *grpE*, *dnaK*, *dnaJ*, *groES*, and *groEL* after heat shock of the wild-type ATCC 3502 strain and the *hrcA* mutant, a volume of 1 ml of the second overnight culture of each strain was used to inoculate 250 ml of buffered TPGY broth each. The cultures were grown anaerobically at 37°C until they reached the mid-exponential growth phase corresponding to an OD_{600} of 0.9 to 1.1 units (pre-heat shock sample, or calibrator sample [t_0]) (Fig. 2). Five-milliliter samples were taken and treated as described above. After sampling, the bottles were tightly closed and placed in a water bath set to 65°C

TABLE 2. Oligonucleotide primers used in the study

Function and name	Sequence (5′–3′)
Intron retargeting	
hrcA-IBS	AAAAAAGCTTATAATTATCCTTAGGTTCCCCACTAGTGCGCCCAGATAGGGTG
hrcA-EBS1d	CAGATTGTACAAATGTGGTGATAACAGATAAGTCCCACTATTTAACTTACCTTTCTTTGT
hrcA-EBS2	TGAACGCAAGTTTCTAATTTCGATTGAACCTCGATAGAGGAAAGTGTCT
dnaK-IBS	AAAAAAGCTTATAATTATCCTTAGCAGCCGTTGGTGTGCGCCCAGATAGGGTG
dnaK-EBS1d	CAGATTGTACAAATGTGGTGATAACAGATAAGTCGTTGGTTCTAACTTACCTTTCTTTGT
dnaK-EBS2	TGAACGCAAGTTTCTAATTTCGATTGCTGCTCGATAGAGGAAAGTGTCT
EBS universal	CGAAATTAGAAACTTGCGTTCAGTAAAC
Intron sequencing	
5402F-F1	TTAAGGAGGTGTATTTCATATGACCATGATTACG
Confirmation of insertion	
hrcA-53-forw	TGGATGAGAGAAAAATCAGG
hrcA-53-rev	TGATGGTTTTCTTCCAGATG
dnaK-440-forw	AGACAAGCGACAAAGGATGC
dnaK-440-rev	TGGTCAAAGTCATCTCCACCT
Gene expression study	
hrcA-forw	ATAGTGGAGAACCCGTTGGA
hrcA-rev	CCATATCTTCTAGGTCTGCCATCT
grpE-forw	AAAGGTAAAGAAGAGGATTTGGAG
grpE-rev	AGACATCTTCACAGGCACTAACA
dnaK-forw	CAGTTATGGAAGGTGGGGAAC
dnaK-rev	GCTTGTCTTTTTGCTACTTGACC
dnaJ-forw	GGATTTGATTCGTCAGGCTTT
dnaJ-rev	GGTCCATTTTTCCTTCTTCCA
groES-forw	CCTGGTGGATTAGTTGATGGA
groES-rev	TCACTTCATTACCGGCATATTTT
groEL-forw	TCAAAATCTATGGGAACTGACC
groEL-rev	AGGCTTCTCCTTCTATGTCTTCA
16S *rrn*-forw	AGCGGTGAAATGCGTAGAGA
16S *rrn*-rev	GGCACAGGGGGAGTTGATAC

together with a temperature control bottle containing a thermometer. The control bottle contained 250 ml of buffered TPGY broth and was kept at 37°C before transfer to the water bath. After the temperature in the control bottle reached 45°C, the cultures were immediately transferred into a 45°C oil bath placed in an anaerobic work station. Samples for RNA isolation and OD measurements were withdrawn immediately after the heat shock (heat shock sample). After heat shock, the cultures were continuously kept at 45°C, and further samples were taken at 20 min, 1 h, 2 h, and 5 h after heat shock. The experiment was carried out in triplicate.

RNA isolation. The frozen cell pellets were resuspended in 0.25 ml of lysis buffer containing 25 mg/ml lysozyme (Sigma-Aldrich, St. Louis, MO) and 250 IU/ml mutanolysin (Sigma-Aldrich) in Tris-EDTA buffer (pH 8.0; Fluka, Biochemica, Switzerland) and incubated for 30 min at 37°C. Total RNA purification was performed using a commercial spin column system (RNeasy Mini Kit; Qiagen, Hilden, Germany) according to the manufacturer's instructions using 700 μl of RLT buffer for lysis, the optional additional drying step, and an on-column DNase treatment (RNase-Free DNase Set; Qiagen). The RNA was eluted twice with 40 μl of DNase- and RNase-free water (Sigma-Aldrich) and subjected to a second DNase treatment with an Ambion DNA-free kit (Applied Biosystems, Life Technologies Corporation, Carlsbad, CA) according to the manufacturer's instructions. The total RNA concentration was determined by measurement of the absorption units at the wavelength of 260 nm (A_{260}) using a

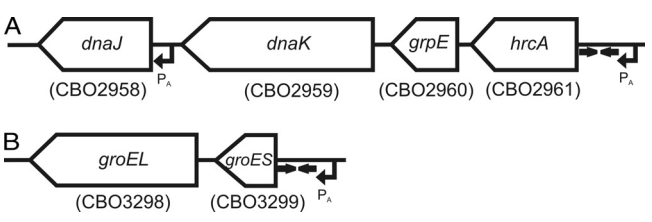

FIG. 1. Organization of the genes studied in the *dnaK* operon (A) and *groE* operon (B) of *C. botulinum* strain ATCC 3502 (20).. The inverted arrows indicate the CIRCE binding element of the negative regulator HrcA. P_A indicates potential σ^A-dependent promoter.

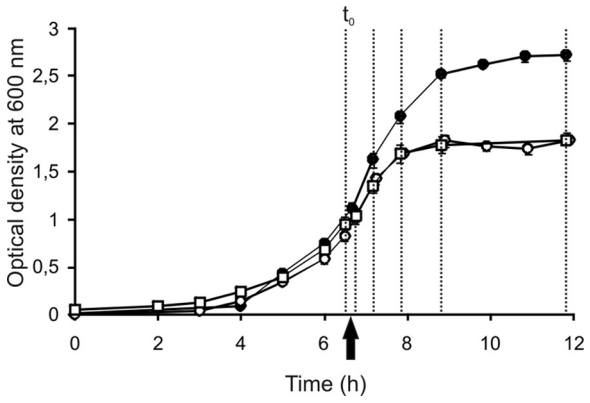

FIG. 2. Growth curves obtained in the gene expression experiments of the *C. botulinum* ATCC 3502 wild type grown at 37°C (filled circles), the *C. botulinum* ATCC 3502 wild type exposed to heat shock to 45°C (open circles), and the *hrcA* mutant exposed to heat shock to 45°C (open squares). Time zero (t_0) is the time when the calibrator sample was withdrawn in the mid-exponential growth phase (OD_{600} of 0.9 to 1.1). The arrow indicates the time of the heat shock, and the dashed lines indicate times of sampling: immediately and 20 min, 1 h, 2 h, and 5 h after heat shock and at the corresponding time points for the untreated culture. The error bars indicate the variations of three biological replicates.

NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific, Waltham, MA). The RNA extracts with an A_{260}/A_{280} ratio of around 2 and an A_{260}/A_{230} ratio higher than 1.8 (considered to be pure RNA) were further examined for RNA degradation with a 2100 Bioanalyzer using Prokaryote Total RNA Nano Chips (Agilent Technologies, Santa Clara, CA). Only RNA samples showing no signs of RNA degradation were used for the reverse transcription (RT) reaction.

Reverse transcription. An amount of 800 ng of total RNA was reverse transcribed into cDNA using a DyNAmo cDNA Synthesis Kit (Finnzymes, Espoo, Finland). The RNA was diluted with water to a volume of 7 μl; 1 μl of random hexamer primers (300 ng/μl; included in the kit) was added, and the mixture was predenatured at 65°C for 5 min. A volume of 10 μl of 2× RT buffer (containing deoxynucleoside triphosphates [dNTPs]) and 2 μl of Moloney murine leukemia virus (M-MuLV) RNase H+ reverse transcriptase were added, and cDNA was synthesized at 37°C according to the manufacturer's instructions. The RT reaction was carried out in duplicate for each RNA sample. A control in which the RT was replaced with 2 μl of water (minus-RT control) was performed to control each RNA sample for possible DNA contamination. The cDNA samples were stored at −70°C until further use.

Quantitative real-time PCRs and relative gene expression analysis. DyNAmo Flash SYBR green quantitative PCR (qPCR) chemistry (Finnzymes) was used for qPCRs performed in a Rotor Gene 3000 Real Time Thermal Cycler (Qiagen) using the following cycling protocol: polymerase activation at 95°C for 1 min, 40 cycles at 95°C for 10 s and 60°C for 20 s with data collection at the end of each cycle, and a final extension step for 1 min at 60°C, followed by a melt curve analysis (from 60°C to 98°C in 0.5°C steps for 10 s). Four microliters of template was amplified in a 20-μl reaction volume containing primers at a final concentration of 0.5 μM, 10 μl of 2× DyNAmo Flash SYBR Green Master Mix (contains the hot start version of a modified *Tbr* DNA polymerase, SYBR green I, optimized PCR buffer, 5 mM $MgCl_2$, dNTP mix including dUTP), and water. The primers for the six class I heat shock genes studied (*hrcA*, *grpE*, *dnaK*, *dnaJ*, *groES*, and *groEL*) and for the 16S *rrn* were designed based on the published ATCC 3502 genome sequence (EMBL accession number AM412317) (21) using the Primer3-web-0.4.0 Web interface (http://primer3.sourceforge.net/webif.php) (Table 2).

Standard curves were created using a 10-fold dilution series of pooled cDNAs to determine the amplification efficiency of each primer pair. For each primer pair, qPCRs were carried out in triplicate for each cDNA dilution. The reaction efficiency and the quantification thresholds for detection of fluorescence above background were automatically calculated using Rotor Gene 3000 software, version 6.1. In each qPCR run, nontemplate controls, containing 4 μl of water replacing the cDNA, were performed to control DNA contamination of the reagents. To check for DNA contamination of the sample RNA, qPCR with melt curve analysis using the 16S *rrn* primers was run on all minus-RT control reaction mixtures. No specific product was detected in nontemplate or minus-RT controls within 40 amplification cycles.

For each sample taken during the experiments, qPCR was performed using 4 μl of 100-fold (target genes) or 10,000-fold (16S *rrn*) diluted cDNA of both reverse transcription reaction mixtures in duplicate as described above. Relative expression values of the six target genes were calculated with the Pfaffl (14) method using following formula: $R = E_{\text{target}}^{\Delta \text{Cq target (Cq-calibrator} - \text{Cq-sample)}} / E_{\text{reference}}^{\Delta \text{Cq reference (Cq-calibrator} - \text{Cq-sample)}}$, where R is the expression ratio of the target gene expression in the sample relative to the calibrator, E is the amplification efficiency of the reaction, and C_q is the amplification cycle number at which the emitted fluorescence of the reaction exceeds the threshold for quantification. The 16S *rrn* was used as a normalization reference (2). The sample withdrawn in the mid-exponential growth phase (at time zero or before heat shock) was used as the calibrator; thus, the fold change values in expression of each gene during later stages of growth at 37°C (wild-type strain) or after heat shock (wild type and *hrcA* mutant strain) were calculated in relation to the mid-exponential phase. When the mid-exponential gene expression levels of the *hrcA* mutant and wild type were compared, the latter was used as a calibrator.

Growth of the wild-type, *hrcA* mutant, and *dnaK* mutant strains at different temperatures, pH values, and NaCl concentrations. The *C. botulinum* strains were grown anaerobically in 10 ml of deoxygenated, buffered TPGY broth for 24 h at 37°C. A volume of 100 μl of each culture was reinoculated into 10 ml of fresh buffered TPGY broth at 37°C overnight for 16 h.

The second overnight culture was used to inoculate fresh deoxygenated buffered TPGY broth (temperature experiments), buffered TPGY broth with the pH adjusted to pH 5.0, 6.0, 7.0, or 7.6 (pH experiments), or TPGY broth without or with 3 g/liter or 3.5 g/liter added NaCl (VWR International, Belgium) (NaCl experiments) in a ratio of 1:100. A volume of 350 μl of these cultures was transferred into wells of a 100-well microtiter plate and anaerobically incubated in a Bioscreen C Microbiology Reader (Oy Growth Curves AB Ltd., Helsinki, Finland) which was placed in an anaerobic cabinet. The cultures were incubated under continuous shaking at 37°C (temperature, NaCl, and pH experiment), 42°C (temperature experiment), or 45°C (temperature experiment), and their OD_{600} values were automatically measured and recorded at 15-min intervals. The experiments were carried out in three biological replicates, with five (temperature and NaCl experiments) or three (pH experiment) technical replicates each.

Growth curves were constructed by plotting the OD_{600} of each culture against the time normalized to the OD_{600} of uninoculated medium. The maximum growth rate in optical density units per hour (ODU/h) of each culture was calculated by fitting the growth curve to the Baranyi and Roberts model (1) using the DMFit, version 2.0, Microsoft Excel add-in program (Institute of Food Research, Norwich, United Kingdom).

Maximum growth temperatures. To determine the maximum growth temperatures ($T_{\max}$), the ATCC 3502 wild-type, *hrcA*, and *dnaK* mutant strains were grown on TPGY agar plates and incubated in the temperature gradient incubator Gradiplate W10 (BCDE Group, Helsinki, Finland) as described previously (7). The temperature gradient ranged from 42°C to 49°C.

Lethal stress experiment. A volume of 100 μl of the second overnight culture was inoculated into 10 ml of buffered TPGY broth. The strains were grown anaerobically at 37°C for 6.0 h (wild type), 6.5 h (*hrcA* mutant), or 8.0 h (*dnaK* mutant) to reach the mid-exponential growth phase. One milliliter of the culture was withdrawn for enumeration using the three-tube most probable number method, and the tubes were sealed. The sealed cultures were placed into a 64°C water bath and heat treated for 2 min, measured from the time at which the temperature in a control tube containing 9 ml of buffered TPGY broth reached 62°C.

The cultures were transferred back to 37°C in the anaerobic chamber immediately after the heat treatment and enumerated. Dilutions for enumeration were prepared in peptone water (0.1% peptone, 0.85% NaCl) and inoculated into 10 ml of TPGY broth. To ensure identical temperature conditions, the experiment was carried out simultaneously for all strains, with three biological replicates. The log reduction in cell number was calculated for each culture to determine the heat tolerance of the strains. When no growth was detected, half of the cell concentration of the detection limit was used.

Statistics. SPSS software, version 15.0.1 (SPSS Inc., Chicago, IL), was used to conduct the statistical analysis. A one-sample t test was used to test if the relative gene expression ratio R (as described above) differed from 1 (no change in expression). An unpaired two-sample t test was applied to test differences between the wild type and the mutants in maximum growth rate, $T_{\max}$, and log reduction after Levene's test was performed for equality of variances.

RESULTS

Relative expression of *hrcA*, *grpE*, *dnaK*, *dnaJ*, *groES*, and *groEL*. In the initial experiments, mid-exponential cultures of the ATCC 3502 wild type and *hrcA* mutant were exposed to heat shock (from 37°C to 45°C), and incubation was continued at 45°C. Compared to the untreated wild-type culture, the wild-type and the *hrcA* mutant strains subjected to the temperature upshift entered stationary phase early and achieved low final cell densities (Fig. 2).

When grown at 37°C, the wild-type strain did not show any major changes in relative expression levels of the genes studied during exponential growth and the transition growth phase in relation to the calibrator sample (Fig. 3). However, all six measured genes were downregulated in the early stationary and stationary growth phases (Fig. 3).

Immediately upon exposure of the wild-type strain to heat shock at 45°C, the relative expression levels of the *dnaK* and *groE* operons were upregulated more than 3- and 11-fold, respectively, in relation to 37°C (Fig. 4A). Both operons continued to be significantly upregulated at the 20-min time point ($P < 0.05$). One hour after heat shock, while the relative expression level of the *dnaK* operon was similar to that observed prior to heat shock, the *groE* operon remained more than 5-fold upregulated ($P < 0.05$) (Fig. 4A). At the two later

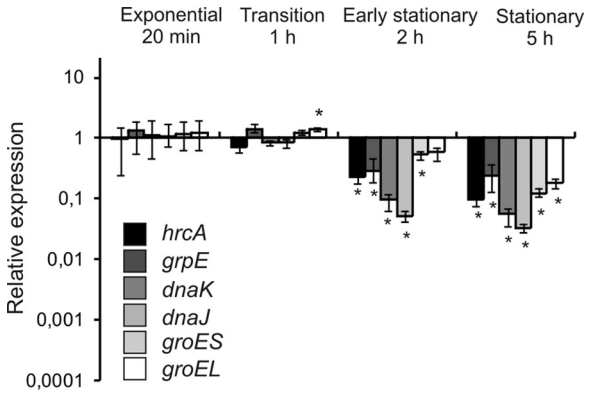

FIG. 3. Relative expression ratios of *hrcA*, *grpE*, *dnaK*, *dnaJ*, *groES*, and *groEL* at different growth phases of *C. botulinum* ATCC 3502 wild type grown at 37°C compared to mid-exponential growth. The 16S *rrn* was used as a normalization reference. The error bars indicate the variations of three biological replicates. Relative expression ratios that differ significantly from 1 ($P < 0.05$) are marked with an asterisk.

time points (2 h and 5 h), downregulation of all genes monitored was more prominent in the heat-treated cultures than at the corresponding time points in the untreated cultures (Fig. 3 and 4A).

It was apparent that even in the absence of heat shock, the expression of the six class I HSGs being monitored was affected by the inactivation of *hrcA*. Thus, during growth at 37°C, all six were more than 2-fold upregulated relative to levels in the wild

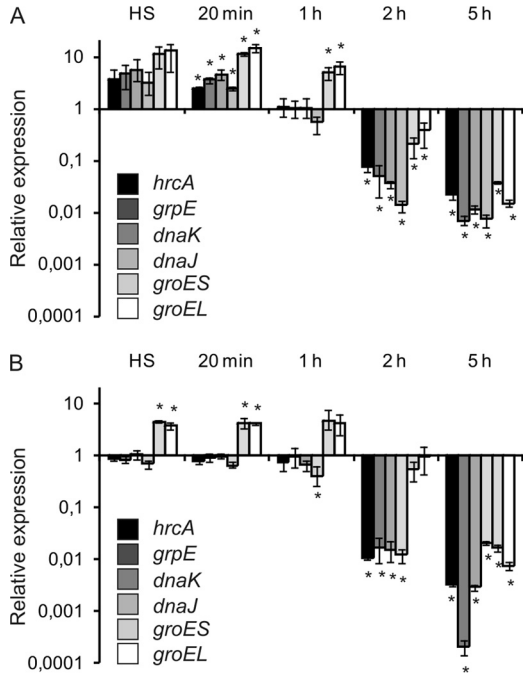

FIG. 4. Relative expression ratios of *hrcA*, *grpE*, *dnaK*, *dnaJ*, *groES*, and *groEL* at different time points after heat shock (HS) at 45°C compared to pre-heat shock, mid-exponential growth of the *C. botulinum* ATCC 3502 wild type (A) and the *hrcA* mutant at 37°C (B). The 16S *rrn* was used 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.

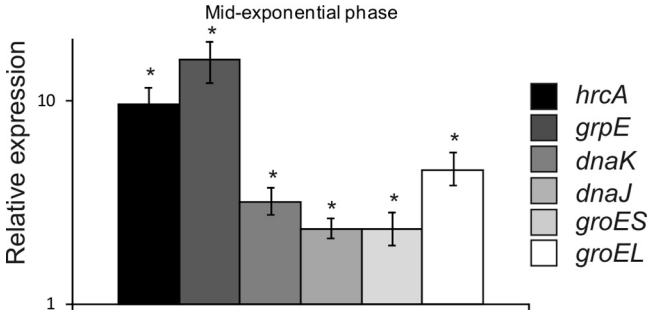

FIG. 5. Relative expression ratios of *hrcA*, *grpE*, *dnaK*, *dnaJ*, *groES*, and *groEL* in the mid-exponential growth phase of the *hrcA* mutant compared to the wild type at 37°C. The 16S *rrn* was used as a normalization reference. The error bars indicate the variations of three biological replicates. Relative expression ratios that differ significantly from 1 ($P < 0.05$) are marked with an asterisk.

type by the mid-exponential phase of growth ($P < 0.05$) (Fig. 5). After heat shock, no significant upregulation of the *dnaK* operon was observed in the *hrcA* mutant ($P > 0.05$), whereas the relative expression of the *groE* operon increased more than 4-fold ($P < 0.05$) and remained at such a level for 1 h (Fig. 4B). At the later time points, the six genes studied were downregulated compared to the pre-heat shock culture at the mid-exponential growth phase.

Growth of the ATCC 3502 wild-type, *hrcA* mutant, and *dnaK* mutant strains at different temperatures, pH values, and NaCl concentrations. The *hrcA* mutant showed slightly reduced growth compared to the wild type when grown at 37°C in buffered TPGY broth at pH 7.0 (Fig. 6A). Accordingly, the maximum growth rate of the wild type was 1.1-fold higher than that of the *hrcA* mutant ($P < 0.05$) (Table 3). No significant difference between maximum growth rates of the wild type and the *hrcA* mutant was observed during growth at 42°C ($P > 0.05$) (Table 3 and Fig. 6B), whereas growth at 45°C resulted in 1.2-fold difference between their maximum growth rates ($P < 0.05$) (Fig. 6C). During growth at pH 6.0, no significant difference between maximum growth rates of the wild type and the *hrcA* mutant was observed ($P > 0.05$) (Fig. 6E). However, at pH 5.0 the largest difference in growth rates between the wild type and *hrcA* mutant was found ($P < 0.05$) (Table 3 and Fig. 6D). The addition of 3% or 3.5% NaCl to the medium at 37°C and pH 7.0 led to a low growth rate and reduced final turbidity of the *hrcA* mutant compared to the wild type (Fig. 6A, G, and H).

The *dnaK* mutant showed reduced growth compared to the wild-type strain under all tested conditions ($P < 0.05$) (Table 3 and Fig. 6A to H). More specifically, when grown in buffered TPGY broth at pH 7.0 and at 37°C, the parent strain grew with a 1.6-fold higher maximum growth rate than the *dnaK* mutant (Table 3 and Fig. 6A). The difference in maximum growth rates became most significant at 42 and 45°C (up to 13-fold difference at 45°C) (Fig. 6C). At pH 6.0 and pH 7.6 the difference in growth performance between the *dnaK* mutant and the wild type was similar to the difference at pH 7.0 (Table 3 and Fig. 6A, E, and F). However, a pH of 5.0 prevented the growth of the *dnaK* mutant almost entirely (Fig. 6D). Increasing concentrations of NaCl in the medium at 37° and pH 7.0 resulted in an increasing difference in the maximum growth rates of the

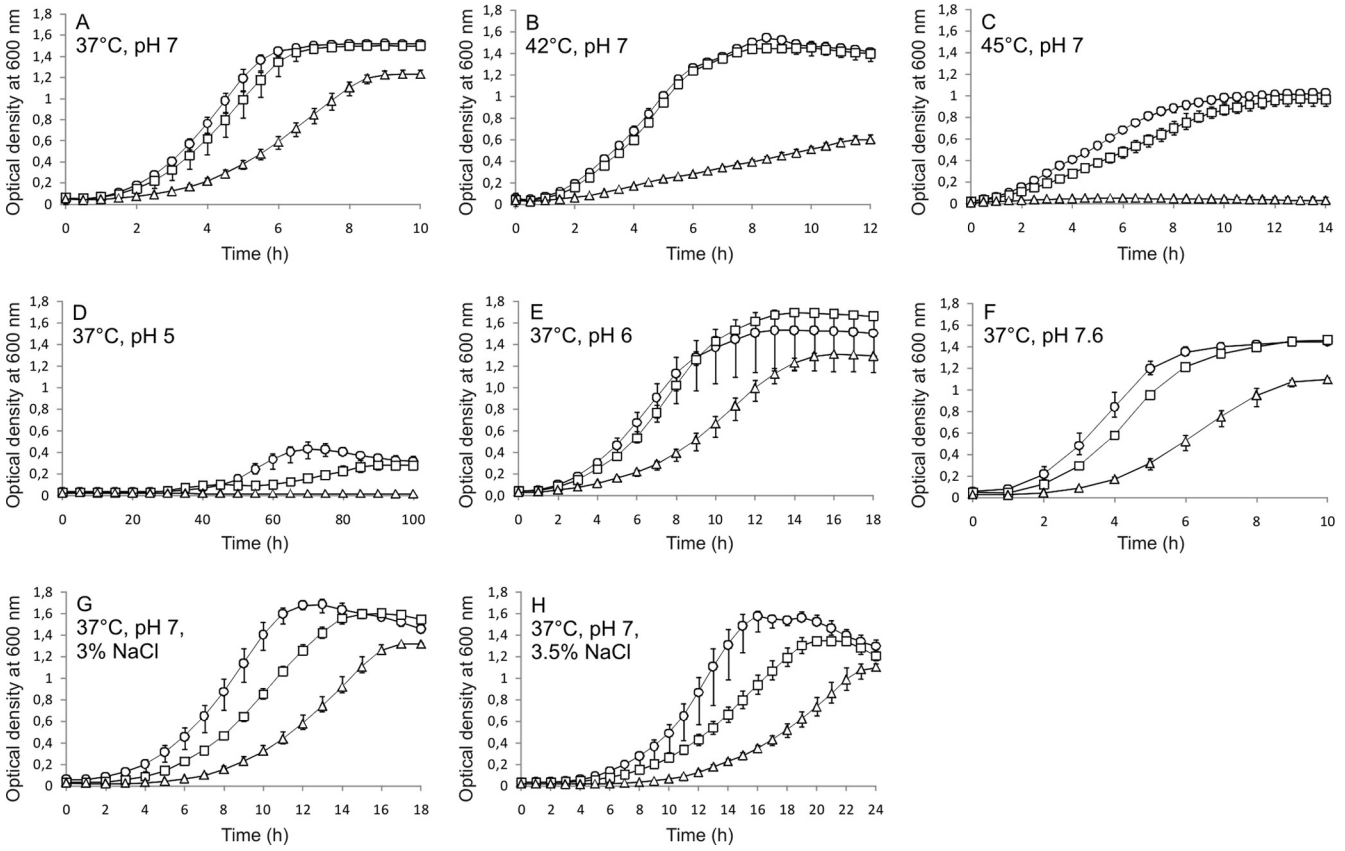

FIG. 6. Growth curves of the *C. botulinum* ATCC 3502 wild type (open circles), *hrcA* mutant (open squares), and *dnaK* mutant (open triangles) at the indicated pH values and temperatures in buffered TPGY broth (A to F) or in TPGY broth with added NaCl (G and H). The error bars indicate the variations of three biological replicates.

dnaK mutant compared to the wild-type strain (Table 3 and Fig. 6G and H).

Maximum growth temperatures. The T_{max} of the *hrcA* mutant was 0.9°C lower ($P < 0.05$) and that of the *dnaK* mutant was 5.1°C lower ($P < 0.05$) than the T_{max} of the wild-type strain (Fig. 7).

Lethal heat stress. After heat treatment of the cultures for 2 min in a 64°C water bath, the number of viable *C. botulinum* ATCC 3502 wild-type cells was reduced by 3.6 log. The *hrcA* mutant showed increased sensitivity to lethal heat stress, with an average reduction of 5.1 log, and the *dnaK* mutant revealed significantly ($P < 0.05$) increased sensitivity, with an average reduction of 8.2 log, compared to the wild-type strain (Fig. 8).

DISCUSSION

The exposure of *C. botulinum* strain ATCC 3502 at the mid-exponential growth phase to heat shock from 37°C to 45°C did not lead to growth arrest (Fig. 2), which contrasts with the reported growth delay of 4 h observed when B. *subtilis* was

TABLE 3. Maximum growth rates of the *C. botulinum* ATCC 3502 wild type, *hrcA* mutant, and the *dnaK* mutant under different growth conditions

Growth conditions	Maximum growth rate (ODU/h ± SD)[a]		
	C. botulinum ATCC 3502	*C. botulinum* ATCC 3502 *hrcA*	*C. botulinum* ATCC 3502 *dnaK*
37°C, pH 7	0.413 ± 0.014	0.380 ± 0.006*	0.257 ± 0.005*
42°C, pH 7	0.305 ± 0.007	0.314 ± 0.009	0.061 ± 0.004*
45°C, pH 7	0.132 ± 0.006	0.108 ± 0.006*	0.010 ± 0.001*
37°C, pH 5	0.024 ± 0.003	0.006 ± 0.002*	ND
37°C, pH 6	0.236 ± 0.001	0.230 ± 0.005	0.156 ± 0.003*
37°C, pH 7.6	0.361 ± 0.004	0.323 ± 0.004*	0.220 ± 0.012*
37°C, pH 7, 3% added NaCl	0.264 ± 0.006	0.203 ± 0.007*	0.155 ± 0.012*
37°C, pH 7, 3.5% added NaCl	0.213 ± 0.008	0.133 ± 0.005*	0.097 ± 0.015*

[a] The maximum growth rates of the mutants significantly different ($P < 0.05$) from the growth rate of the *C. botulinum* wild-type strain are marked with an asterisk. ND, not determined; ODU, optical density units.

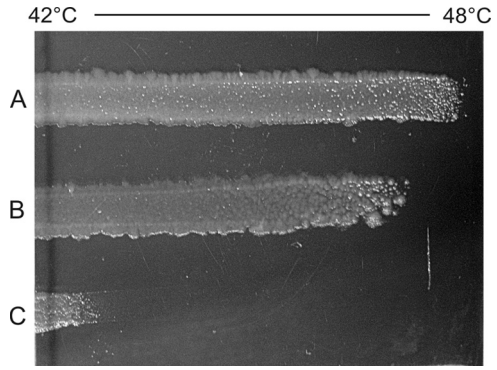

FIG. 7. The cultures of the *C. botulinum* ATCC 3502 wild type (A), *hrcA* mutant (B), and *dnaK* mutant (C) grown for 24 h on TPGY plates in a temperature gradient in the Gradiplate W10 incubator.

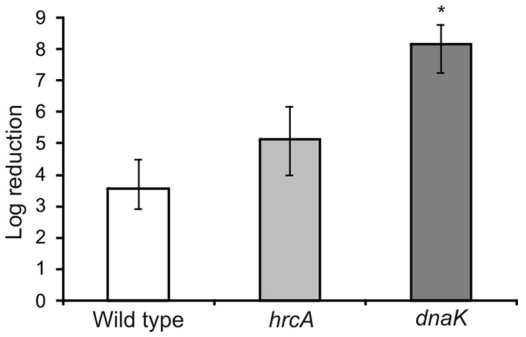

FIG. 8. Log reduction of the cell number of the wild type (white), *hrcA* mutant (light gray), and *dnaK* mutant (dark gray) after heat treatment for 2 min in a 64°C water bath. The error bars indicate the variations of three biological replicates. The log reduction of a mutant significantly different ($P < 0.05$) from that of the *C. botulinum* wild-type strain is marked with an asterisk.

exposed to heat shock (18). Not even a temperature upshift to 49°C led to immediate cessation of growth but resulted in earlier entry into the stationary growth phase at lower cell density than with heat shock to 45°C (data not shown). This suggests that *C. botulinum* possesses rapid and effective measures to overcome temperature stress. Interestingly, no evidence for the presence of a σ^B-dependent general stress regulon, which plays a major role in the heat shock and general stress response of *B. subtilis* and many other Gram-positive bacteria (6), can be found in the *C. botulinum* ATCC 3502 genome (21).

Heat shock from 37°C to 45°C induced transcription of the *dnaK* and *groE* operons in *C. botulinum*. While the relative expression levels of the *dnaK* operon genes returned to pre-heat shock levels 1 h after heat shock, the more prominently induced *groE* operon remained upregulated for a longer time period. A transient increase in the expression of both operons has also been reported for *B. subtilis* (17, 26). However, we found that the class I HSGs of *C. botulinum* were upregulated for considerably longer following heat shock than those of *B. subtilis*. For example, the *dnaK* operon was significantly induced at least 20 min after heat shock in *C. botulinum*, while in *B. subtilis* induction lasted for less than 10 min (26). Also *Clostridium acetobutylicum*, another *Clostridium* species lacking a σ^B regulon, shows transient and, compared to *B. subtilis*, prolonged heat induction of the *dnaK* and *groE* operons (10, 11). We thus suggest that the continued expression of class I HSGs may contribute to the greater ability of *C. botulinum* and *C. acetobutylicum* than *B. subtilis* to recover from heat shock (18).

Without changes in temperature, the six class I HSGs monitored did not significantly change their relative expression levels during exponential growth of the *C. botulinum* wild-type strain but showed clear downregulation in the transition and stationary growth phases. This emphasizes the importance of molecular chaperones in assisting protein folding, especially during rapid cell growth when large amounts of proteins are being newly synthesized and need to undergo folding (3).

The inactivation of *hrcA* caused a significantly higher basal relative expression level of class I HSGs under nonstressed conditions than the wild-type strain, confirming the role of the encoded HrcA as a repressor of class I HSGs in *C. botulinum*, as in other Gram-positive bacteria (8, 18). After heat shock to 45°C no significant change in relative expression levels of the *dnaK* operon was detected in the *hrcA* mutant strain. This can be explained by the *dnaK* operon already being expressed at a high level in the absence of HrcA, such that further upregulation is impossible or unnecessary. Nevertheless, both *groE* operon genes were significantly induced after heat shock. A similar observation in *B. subtilis* led to the assumption that an additional unknown regulatory mechanism exists for the *groE* operon (18). Sagane et al. (16) reported a putative binding site for the σ^K subunit of RNA polymerase in addition to the σ^A binding site upstream of the CIRCE element of the *groE* operon in a type D *C. botulinum* strain and suggested a possible link between spore formation and *groE* operon expression. However, using the bacterial sigma70 promoter recognition software BPROM freeware (Softberry, Mount Kisco, NY), only one promoter with a σ^A binding site was predicted upstream of the *groE* operon of *C. botulinum* strain ATCC 3502. Also for *B. subtilis* a DBTBS database (24) search gave no indication that σ^K contributes to the regulation of the *groE* operon.

The growth studies on the *hrcA* and *dnaK* mutants showed that both strains were viable under a wide range of growth conditions. The *hrcA* mutant showed a mild reduction in growth rate at 37°C and pH 7.0 compared to the wild-type strain. However, under conditions of mild stress (42°C or pH 6.0), no significant difference in growth between the two strains could be observed. The continuous overexpression of class I HSGs may lead to a constant high concentration of molecular chaperones in the cell, supporting growth under moderately nonoptimal conditions. This may compensate for the additional metabolic burden of deregulated expression of the *dnaK* and *groE* operons, a possible reason for growth slower than that of wild type under optimal conditions. This conclusion is in agreement with a report that a *B. subtilis* Δ*hrcA* mutant resumed growth faster after heat shock than the parental strain (18). However, compared to the wild-type strain, the *hrcA* mutant showed restricted growth when challenged by more severe stresses, reflected in reduced growth rates at 45°C, pH 5.0, and in the presence of increasing NaCl concentrations. Furthermore, the *hrcA* mutant exhibited a higher reduction in

cell number after exposure to lethal heat stress at 64°C and a lower maximum growth temperature than the wild type. These findings highlight the need for intact regulatory mechanisms for class I HSGs for *C. botulinum* to tolerate stressful environmental conditions by means of adaptation of the molecular chaperone concentration in the cytosol.

Compared to the wild type and in contrast to the *hrcA* mutant, the *dnaK* mutant showed clearly reduced growth under optimal conditions (37°C and pH 7.0). This finding emphasizes the importance of the molecular chaperone DnaK for *C. botulinum* to assist protein folding under not only stressful environmental conditions but also normal physiological conditions. The impaired growth of the *dnaK* mutant was more pronounced at a high temperature, as demonstrated by the 5°C lower maximum growth temperature than that of the wild type and declining maximum growth rates at increasing temperatures. Deficiency in growth at optimal and high temperatures has also been reported for *E. coli* and *B. subtilis* carrying mutations in the *dnaK* gene (13, 19). The major role of functional DnaK for the heat resistance of *C. botulinum* was also reflected by the more than 4-log-higher reduction in viable cell number of the *dnaK* mutant than that of the wild-type strain after exposure to lethal heat stress.

The fact that an increasing NaCl concentration, and especially a low pH of 5.0, resulted in noticeable impairment of growth of the *hrcA* mutant and particularly the *dnaK* mutant compared to the wild type demonstrates that class I HSGs and especially the molecular chaperone DnaK play an important role in *C. botulinum* in response to not only heat but also NaCl or acid stress. Involvement of these genes of *C. botulinum* in the response to other stresses, which also lead to the accumulation of nonnative protein in the cytosol, is likely.

C. botulinum, whether growing in food products, the intestinal tract (in cases of infant or intestinal botulism), or wounds, encounters changes in environmental temperatures, low pH, high NaCl concentrations, and other stresses. Our results demonstrate that the function of class I HSGs and of the molecular chaperone DnaK is necessary to allow the bacterium to survive these challenges. Class I HSGs permit growth and may thus eventually enhance the toxic potential of *C. botulinum* under stressful conditions, thereby contributing to its hazardous nature.

ACKNOWLEDGMENTS

This work was performed in the Centre of Excellence in Microbial Food Safety Research, Academy of Finland (grants 118602, 1115133, and 1120180). The work was also supported by the Finnish Funding Agency for Technology and Innovation (TEKES) (2431/31/04), the Finnish Ministry of Agriculture and Forestry (4655/501/2003, 4541/501/2006), the Finnish Graduate School on Applied Bioscience, the Walter Ehrström Foundation, and the Finnish Veterinary Foundation.

We thank Esa Penttinen, Hanna Korpunen, Kirsi Ristkari, and Heimo Tasanen for their technical assistance.

REFERENCES

1. **Baranyi, J., and T. Roberts.** 1994. A dynamic approach to predicting bacterial growth in food. Int. J. Food Microbiol. **23:**277–294.
2. **Couesnon, A., S. Raffestin, and M. R. Popoff.** 2006. Expression of botulinum neurotoxins A and E, and associated non-toxin genes, during the transition phase and stability at high temperature: analysis by quantitative reverse transcription-PCR. Microbiology **152:**759–770.
3. **Hartl, F. U., and M. Hayer-Hartl.** 2002. Molecular chaperones in the cytosol: from nascent chain to folded protein. Science **295:**1852–1858.
4. **Hatheway, C. L.** 1992. *Clostridium botulinum* and other clostridia that produce botulinum neurotoxin, p. 3–20. *In* A. H. D. Hauschild and K. L. Dodds (ed.), *Clostridium botulinum*: ecology and control in foods. Marcel Dekker, New York, NY.
5. **Heap, J. T., O. J. Pennington, S. T. Cartman, G. P. Carter, and N. P. Minton.** 2007. The ClosTron: a universal gene knock-out system for the genus *Clostridium*. J. Microbiol. Methods **70:**452–464.
6. **Hecker, M., J. Pane-Farre, and U. Volker.** 2007. SigB-dependent general stress response in *Bacillus subtilis* and related Gram-positive bacteria. Annu. Rev. Microbiol. **61:**215–236.
7. **Hinderink, K., M. Lindstrom, and H. Korkeala.** 2009. Group I *Clostridium botulinum* strains show significant variation in growth at low and high temperatures. J. Food Prot. **72:**375–383.
8. **Lemos, J. A. C., Y. Y. M. Chen, and R. A. Burne.** 2001. Genetic and physiologic analysis of the *groE* operon and role of the HrcA repressor in stress gene regulation and acid tolerance in *Streptococcus mutans*. J. Bacteriol. **183:**6074–6084.
9. **Mogk, A., et al.** 1998. Nonnative proteins induce expression of the *Bacillus subtilis* CIRCE regulon. J. Bacteriol. **180:**2895–2900.
10. **Narberhaus, F., and H. Bahl.** 1992. Cloning, sequencing, and molecular analysis of the *groESL* operon of *Clostridium acetobutylicum*. J. Bacteriol. **174:**3282–3289.
11. **Narberhaus, F., K. Giebeler, and H. Bahl.** 1992. Molecular characterization of the *dnaK* gene region of *Clostridium acetobutylicum*, including *grpE*, *dnaJ*, and a new heat shock gene. J. Bacteriol. **174:**3290–3299.
12. **Neidhardt, F., R. VanBogelen, and V. Vaughn.** 1984. The genetics and regulation of heat-shock proteins. Annu. Rev. Genet. **18:**295–329.
13. **Paek, K. H., and G. C. Walker.** 1987. *Escherichia coli dnaK* null mutants are inviable at high temperature. J. Bacteriol. **169:**283–290.
14. **Pfaffl, M. W.** 2001. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res. **29:**2002–2007.
15. **Purdy, D., et al.** 2002. Conjugative transfer of clostridial shuttle vectors from *Escherichia coli* to *Clostridium difficile* through circumvention of the restriction barrier. Mol. Microbiol. **46:**439–452.
16. **Sagane, Y., et al.** 2003. Molecular characterization of GroES and GroEL homologues from *Clostridium botulinum*. J. Protein Chem. **22:**99–108.
17. **Schmidt, A., M. Schiesswohl, U. Volker, M. Hecker, and W. Schumann.** 1992. Cloning, sequencing, mapping, and transcriptional analysis of the *groESL* operon from *Bacillus subtilis*. J. Bacteriol. **174:**3993–3999.
18. **Schulz, A., and W. Schumann.** 1996. *hrcA*, the first gene of the *Bacillus subtilis dnaK* operon encodes a negative regulator of class I heat shock genes. J. Bacteriol. **178:**1088–1093.
19. **Schulz, A., B. Tzschaschel, and W. Schumann.** 1995. Isolation and analysis of mutants of the *dnaK* operon of *Bacillus subtilis*. Mol. Microbiol. **15:**421–429.
20. **Schumann, W.** 2003. The *Bacillus subtilis* heat shock stimulon. Cell Stress Chaperones **8:**207–217.
21. **Sebaihia, M., et al.** 2007. Genome sequence of a proteolytic (Group I) *Clostridium botulinum* strain Hall A and comparative analysis of the clostridial genomes. Genome Res. **17:**1082–1092.
22. **Shukla, H. D., and B. R. Singh.** 2009. Hemagglutinin-33 in the neurotoxin complex of type A *Clostridium botulinum* is a heat shock protein. Botulinum J. **1:**309–317.
23. **Shukla, H. D., and B. R. Singh.** 1999. Identification of DnaJ-like chaperone in *Clostridium botulinum* type A. J. Protein Chem. **18:**695–700.
24. **Sierro, N., Y. Makita, M. De Hoon, and K. Nakai.** 2008. DBTBS: a database of transcriptional regulation in *Bacillus subtilis* containing upstream intergenic conservation information. Nucleic Acids Res. **36:**D93–D96.
25. **Singh, V. K., et al.** 2007. Role for *dnaK* locus in tolerance of multiple stresses in *Staphylococcus aureus*. Microbiology **153:**3162–3173.
26. **Wetzstein, M., et al.** 1992. Cloning, sequencing, and molecular analysis of the *dnaK* locus from *Bacillus subtilis*. J. Bacteriol. **174:**3300–3310.