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The CLO3403/CLO3404 Two-Component System of *Clostridium botulinum* E1 Beluga Is Important for Cold Shock Response and Growth at Low Temperatures

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In order to survive a temperature downshift, bacteria have to sense the changing environment and adjust their metabolism and structure. Two-component signal transduction systems (TCSs) play a central role in sensing and responding to many different environmental stimuli. Although the nonproteolytic (group II) *Clostridium botulinum* represents a major hazard in chilled foods, the cold adaption mechanisms of group II *C. botulinum* organisms are not known. Here, we show that the CLO3403/CLO3404 TCS of *C. botulinum* E1 Beluga is involved in the cold shock response and growth at 12°C. Cold shock induced the expression of the genes encoding the histidine kinase (*clo3403*) and the response regulator (*clo3404*) by more than 100-fold after 5 h relative to their expression in a nonshocked culture at the corresponding time point. The involvement of CLO3403/CLO3404 in growth at low temperature was demonstrated by impaired growth of the insertional *clo3403* and *clo3404* knockout mutants at 12°C compared to the growth of the wild-type culture. Additionally, the inactivation of *clo3403* had a negative effect on motility. The growth efficiency at 12°C of the TCS mutants and the motility of the kinase mutants were restored by introducing a plasmid harboring the operon of the CLO3403/CLO3404 TCS. The results suggest that the CLO3403/CLO3404 TCS is important for the cold tolerance of *C. botulinum* E1 Beluga.

Group II (nonproteolytic) *Clostridium botulinum* type E is a notorious food-borne pathogen which is mainly found in aquatic environments in cold regions of the world (e.g., Northern Europe, Japan, Canada, Alaska, and Greenland) (1, 2), with a high prevalence of spores in the Baltic Sea (3). Type E botulism is usually caused through consumption of contaminated fish or seafood products such as vacuum-packed smoked fish, salted fish, and fermented marine mammals (4–11). Concerning modern food processing, *C. botulinum* type E is the principal food safety hazard in refrigerated, minimally processed packaged foods of aquatic origin (12). The mild heat treatments used in the production of these foods are not sufficient to eliminate all spores, and the limited use of salt and preservatives, as well as vacuum packaging, may support growth and toxin production by *C. botulinum*. Most importantly, thermal control is often not sufficient to prevent food poisoning, as some strains of group II *C. botulinum* are able to grow and produce toxin at temperatures as low as 3°C (13–15).

The mechanisms of cold shock response and growth at low temperature have been thoroughly studied in the model organisms *Escherichia coli* and *Bacillus subtilis* (16–18) but are still unknown in the psychrotrophic *C. botulinum* type E. The cold shock proteins (Csps) are a universal group of cold-induced proteins occurring in a wide range of bacteria and other organisms (17, 19). While group I *C. botulinum* strains carry Csp genes in their genome (20), none were found in the genomes of *C. botulinum* type E strains (21), which raises the question of alternative strategies for coping with cold by *C. botulinum* type E. It is known that two-component signal transduction systems (TCSs) play a role in the cold tolerance and cold shock response of various bacteria (22–26), and recent studies have shown the importance of two TCSs in the cold adaption of the mesophilic group I *C. botulinum* (27, 28). Typical TCSs consist of a sensory histidine kinase and a response regulator. The histidine kinase is often embedded in the cell membrane, sensing a designated stimulus with its N-terminal sensor domain. When stimulated, the C-terminal histidine residue in the cytoplasmic kinase domain becomes autophosphorylated and the phosphoryl group is transferred to the N-terminal aspartate residue in the receiver domain of the cognate intracellular response regulator. Furthermore, the phosphoryl group is transferred to the C-terminal output domain. Response regulators control the transcription of their target genes through specific DNA-binding activity. TCSs respond to many environmental stimuli, including pH, osmolarity, oxidative stress, and temperature (29–31). The best-characterized bacterial TCS contributing to cold tolerance is the DesK/DesR of *B. subtilis*. DesK/DesR responds to decreased membrane fluidity caused by a temperature downshift and induces the transcription of *des*. The *des*-encoded desaturase restores membrane fluidity at low temperatures by increasing the desaturation of the acyl chains of membrane phospholipids (22, 32–37). Whether and how TCSs in group II *C. botulinum* organisms respond to low temperatures is not known.

Here, we show that the CLO3403/CLO3404 TCS of *C. botulinum* E1 Beluga is involved in the cold shock response and growth at 12°C. The relative mRNA levels of *clo3403* and *clo3404* were induced after a cold shock (temperature downshift from 30°C to 12°C), and insertional knockout mutants with disrupted *clo3403* and *clo3404* showed a cold-sensitive phenotype. The mutations were successfully complemented by introducing a plasmid harboring the operon of the CLO3403/CLO3404 TCS.

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TABLE 1 Strains and plasmids used in this study

Strain description or plasmid	Description	Source or reference
C. botulinum strains		
E1 Beluga	Wild-type strain (WT)	IFR[a]
clo3403$_s$	Insertional mutation of *clo3403* in sense direction at position 630-631	This study
clo3403$_a$	Insertional mutation of *clo3403* in antisense direction at position 227-228	This study
clo3404$_s$	Insertional mutation of *clo3404* in sense direction at position 384-385	This study
clo3404$_a$	Insertional mutation of *clo3404* in antisense direction at position 505-506	This study
WT-pMTL82151	WT strain with empty plasmid	This study
clo3403$_s$-pMTL82151	*clo3403*$_s$ mutant with empty plasmid	This study
clo3403$_a$-pMTL82151	*clo3403*$_a$ mutant with empty plasmid	This study
clo3404$_s$-pMTL82151	*clo3404*$_s$ mutant with empty plasmid	This study
clo3404$_a$-pMTL82151	*clo3404*$_a$ mutant with empty plasmid	This study
WT-pMTL82151::*clo3404-clo3401*	WT strain with complementation plasmid	This study
clo3403$_s$-pMTL82151::*clo3404-clo3401*	*clo3403*$_s$ mutant with complementation plasmid	This study
clo3403$_a$-pMTL82151::*clo3404-clo3401*	*clo3403*$_a$ mutant with complementation plasmid	This study
clo3404$_s$-pMTL82151::*clo3404-clo3401*	*clo3404*$_s$ mutant with complementation plasmid	This study
clo3404$_a$-pMTL82151::*clo3404-clo3401*	*clo3404*$_a$ mutant with complementation plasmid	This study
E. coli strains		
CA434	Conjugation donor	44
NEB 5-alpha competent	Cloning strain	New England BioLabs
Plasmids		
pMTL007C-E2	ClosTron vector for mutagenesis	42
pMTL007C-E2::*clo3403*-631s	pMTL007C-E2 targeting *clo3403* in sense direction	This study (DNA2.0)
pMTL007C-E2::*clo3403*-228a	pMTL007C-E2 targeting *clo3403* in antisense direction	This study (DNA2.0)
pMTL007C-E2::*clo3404*-385s	pMTL007C-E2 targeting *clo3404* in sense direction	This study (DNA2.0)
pMTL007C-E2::*clo3404*-506a	pMTL007C-E2 targeting *clo3404* in antisense direction	This study (DNA2.0)
pMTL82151	Plasmid vector	45
pMTL82151::*clo3404-clo3401*	Complementation plasmid containing the operon of the CLO3403/CLO3404 TCS	This study

[a] Culture Collection of the Institute of Food Research, Norwich, United Kingdom.

MATERIALS AND METHODS

Strains, plasmids, and culture. The bacterial strains and plasmids used in this study are listed in Table 1. *C. botulinum* E1 Beluga was used as a wild-type (WT) strain and for constructing insertional *clo3403* and *clo3404* knockout mutants. *C. botulinum* E1 Beluga was initially isolated from a food-borne botulism outbreak in Alaska that was associated with beluga flippers (38). *C. botulinum* strains were grown at 30°C or 12°C in tryptone-peptone-glucose-yeast extract (TPGY) broth (50 g/liter tryptone, 5 g/liter peptone, 4 g/liter glucose, and 20 g/liter yeast extract [Difco, Becton Dickinson, Sparks, MD] plus 1 g/liter sodium thioglycolate [Merck KGaA, Darmstadt, Germany]) or on TPGY agar (1%) supplemented with thiamphenicol (15 μg/ml), cycloserine (250 μg/ml), and erythromycin (2.5 μg/ml) when appropriate (Sigma-Aldrich, St. Louis, MO). Culturing was performed under strictly anaerobic conditions in an anaerobic work station (MG1000 anaerobic work station; Don Whitley Scientific Ltd., Shipley, United Kingdom) with an atmosphere of 85% N_2, 10% CO_2 and 5% H_2. Agar plates and broth were deoxygenated before use by anaerobic storage for 48 h or by boiling for 15 min, respectively. *E. coli* was grown aerobically in Luria-Bertani (LB) broth (Difco) or on LB agar plates at 37°C. When appropriate, chloramphenicol (25 μg/ml) and kanamycin (30 μg/ml) were used for selection (Sigma-Aldrich).

All experiments were performed in triplicate. Three single colonies of each strain (biological replicates) were separately inoculated into 10 ml TPGY broth and incubated at 30°C for 24 h. The cultures were diluted (1:100) into fresh TPGY (10 ml) and incubated at 30°C for 16 h. The second overnight culture (ONC) was used as the starting culture for the experiments.

Cold shock. The *C. botulinum* E1 Beluga ONC was diluted (1:100) into 200 ml of fresh TPGY broth and incubated at 30°C. Growth was tracked by measurement of the optical density at 600 nm (OD_{600}). At an OD_{600} of 1.0 to 1.2, the culture was divided into two 100-ml cultures and one culture was transferred to an ice-water bath. The temperature of the culture was followed by mixing the culture with a sterile thermometer. Reaching 12°C implied cold shock. The culture was incubated at 12°C for 24 h. The other culture represented the nonshocked control and was incubated at 30°C over the same time period. Samples for RNA isolation were taken from cold-shocked and nonshocked cultures immediately before (time zero [t_0]) and 1 min, 30 min, 1 h, 2 h, 5 h, and 24 h after the cold shock (Fig. 1). A volume of 250 μl of an ethanol (99%)–phenol (95%) solution (9:1) was added to 1,250 μl of each sample. The mixture was incubated for 30 min at 4°C, and the cells were pelleted by centrifugation (5 min and 8,000 rpm at 4°C). The cell pellets were stored at −70°C. RNA isolation was performed within 30 days.

RNA isolation. Frozen cell pellets were resuspended in 250 μl of lysis buffer containing lysozyme (50 mg/ml; Sigma-Aldrich), mutanolysin (1 U/ml; Sigma-Aldrich), and Tris-EDTA (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) at a ratio of 2:1:1. The mixture was incubated at 37°C for 2 h. RNA was isolated using the RNeasy minikit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Genomic DNA was removed by DNase treatment (RNase-free DNase set; Qiagen) and, after the final RNA elution, by a second DNase treatment (DNA-*free*; Ambion, Life Technologies Corporation, Carlsbad, CA) (39). The RNA concentration was determined using the NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Inc., Waltham, MA). The RNA quality was checked with the Agilent 2100 bioanalyzer (Agilent Technologies, Santa Clara, CA).

RT. The cDNA was synthesized using the DyNAmo cDNA synthesis kit (Thermo Scientific). A total of 800 ng of RNA was reverse transcribed according to the manufacturer's instructions, with the exception of a predenaturation step for 5 min at 65°C to reduce secondary structures of the

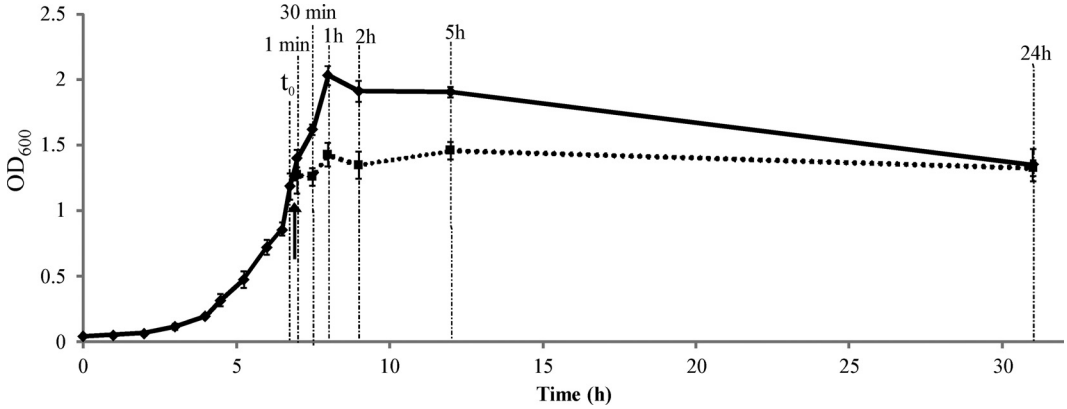

FIG 1 Cold shock of *C. botulinum* E1 Beluga. The *C. botulinum* E1 Beluga ONC was diluted (1:100) into 200 ml of fresh TPGY broth and incubated at 30°C (solid line). During mid-exponential growth phase, half of the culture was cold shocked (indicated by arrow) and growth was continued at 12°C (dotted line). Samples for RT-qPCR analysis were collected from both cultures before (t_0) and 1 min, 30 min, 1 h, 2 h, 5 h, and 24 h after the cold shock (vertical dashed lines). Error bars represent standard deviations of three biological replicates.

RNA template. For each RNA sample, two reactions were performed. DNA contamination was controlled by reactions with no reverse transcriptase. Synthesized cDNA was stored at −20°C before use in quantitative real-time reverse transcription-PCR (RT-qPCR).

RT-qPCR. For RT-qPCR, the Maxima SYBR green qPCR master mix (2×; Thermo Scientific) was used according to the manufacturer's instructions. The PCR mixtures consisted of 1× Maxima SYBR green qPCR master mix, 0.3 μM each primer (Table 2), and 4 μl of 10^{-2}-fold-diluted cDNA (for *clo3403* and *clo3404*) or 10^{-6}-fold-diluted cDNA (for 16S *rrn*) in a total volume of 25 μl. Two PCRs for each cDNA sample (PCR replicates) were performed in the Rotor-Gene 3000 real-time thermal cycler (Qiagen). The run comprised one cycle at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 60 s, and a final cycle at 60°C for 60 s. The PCR efficiencies were determined for each primer pair with serially diluted cDNA pools as the templates. The efficiencies were 0.97 for 16S *rrn*, 0.98 for *clo3403*, and 0.92 for *clo3404*. Melting curve analysis was performed after each run from 60°C to 98°C in 0.5°C steps for 10 s to confirm the specificity of the PCR products. Gene expression was normalized to the expression of 16S *rrn*, and the relative gene expression values were calculated as described by Pfaffl (40). The two-tailed Student's *t* test was used to compare fold changes of relative gene expression.

Construction of insertional *clo3403* and *clo3404* knockout mutants with the ClosTron. The TCS genes *clo3403* and *clo3404* were disrupted by inserting a mobile group II intron from *Lactococcus lactis* (Ll.*ltr*B) using the ClosTron system (41, 42). Target sites were designed using the intron design tool, based on the Perutka algorithm (43), at ClosTron.com (42). For mutagenesis of each gene, two target sites were designed for inserting the intron in the sense orientation (s) of the target gene and in the antisense orientation (a), resulting in two mutants (sense and antisense) for each gene. For *clo3403*, the insertions were targeted in sense orientation to position 630-631 (*clo3403*$_s$) and in antisense orientation to position 227-228 (*clo3403*$_a$). The insertions to *clo3404* were targeted at position 384-385 (*clo3404*$_s$) and at position 505-506 (*clo3404*$_a$). Retargeted pMTL007C-E2 plasmids were ordered from DNA2.0, Inc. (Menlo Park, CA), and transformed by a heat shock into chemically competent *E. coli* CA434 (44). Transformants were grown in 5 ml of LB broth supplemented with kanamycin (30 μg/ml) and chloramphenicol (25 μg/ml). Retargeted plasmids were then conjugated into *C. botulinum* E1 Beluga on TPGY agar for 6 h. Transconjugants were screened on TPGY agar containing thiamphenicol (15 μg/ml) and cycloserine (250 μg/ml) and streaked on TPGY agar containing erythromycin (2.5 μg/ml) for the selection of integrants. Insertion in the correct site and orientation were confirmed by PCR using target-specific intron-flanking primers and the intron-binding EBS Universal primer. Single intron insertion was verified by Southern blotting as described previously (23).

Construction of the complementation plasmid. A 4,660-bp fragment containing *clo3404*, *clo3403*, *clo3402*, and *clo3401*, 457 bp upstream from *clo3404* and 154 bp downstream from *clo3401*, was amplified. This frag-

TABLE 2 Primers used in this study

Primer	Sequence (5′→3′)	Use
clo3403-F	GACAAGGCATTCCGAAAGAA	RT-qPCR
clo3403-R	CAGTAGTTCCATTCAAACTGCTTA	RT-qPCR
clo3404-F	AGCAGAATGTGGGATAGATGC	RT-qPCR
clo3404-R	CAGCACCAAGCTGAAGACCT	RT-qPCR
16S *rrn*-F	AGGAGCAATCCGCTATGAGA	RT-qPCR
16S *rrn*-R	GTGCAATATTCCCCACTGCT	RT-qPCR
clo3403-F2	AGAAAACGTTTTGAATGAGATGAG	Screening for mutants
clo3403-R2	CCTAAAATTGCTGCTAATGGAG	Screening for mutants
clo3404-F2	GTCTTCAGCTTGGTGCTGAC	Screening for mutants
clo3404-R2	ACTATATCCCCATATCTTGTCCAA	Screening for mutants
EBS Universal	CGAAATTAGAAACTTGCGTTCAGTAAAC	Screening for mutants
clo3404-F-NotI	NNNNNNGCGGCCGCGGAAATCGCATTCCTTTCAT	Construction of complementation plasmid
clo3401-R-NdeI	NNNNNNCATATGTGTATTTTATTGTTTGGTTGTGTTTTT	Construction of complementation plasmid
Intron-F	TGGCAATGATAGCGAAACAA	Southern blotting
Intron-R	GGTACCGCCTTGTTCACATT	Southern blotting

ment represents the operon containing the TCS genes (*clo3403* and *clo3404*), an ABC transporter gene (*clo3401*), a gene encoding a conserved hypothetical protein with a predicted transmembrane helix (*clo3402*), and the putative natural promoter of the operon. The fragment was purified with the Gene JET PCR purification kit (Thermo Scientific), digested with NotI and NdeI, and ligated into pMTL82151 (45, 46). The resulting pMTL82151::*clo3404-clo3401* was sequenced, transformed into *E. coli* CA434, and further conjugated into the mutants and the WT strain. As a vector control, the empty pMTL82151 plasmid was conjugated into the mutants and the WT.

Growth experiments at 12°C and 30°C. The *C. botulinum* ONC was diluted (1:100) into 10 ml of anaerobic TPGY broth. Volumes of 350 μl of this dilution were pipetted into four wells (technical replicates) of a 100-well microtiter plate and incubated at 12°C for 10 days or at 30°C for 2 days in the Bioscreen C microbiology reader (Oy Growth Curves AB, Helsinki, Finland) in an anaerobic workstation under continuous shaking (47, 48). The OD_{600} values were measured automatically at 15-min intervals, and growth curves were constructed by plotting the OD_{600} values against time. The maximum growth rate (OD_{600} value per hour) of each culture was calculated by fitting the growth curve to the Baranyi and Roberts model (49) using the DMFit Microsoft Excel add-in program (Institute of Food Research, Norwich, United Kingdom). The mean maximum growth rate of the four technical replicates was determined. Three biological replicates were used for each strain. The two-tailed Student's *t* test was used to compare maximum growth rates.

Motility assay. Single colonies, grown on TPGY agar, were stab inoculated with a sterile inoculating loop into tubes containing TPGY agar (0.3%). The tubes were incubated at 12°C or 30°C under anaerobic conditions. Growth and motility were followed over 7 days and 2 days, respectively. Three biological replicates were used for each strain.

Electron microscopy. The *C. botulinum* ONCs were diluted (1:100) into 50 ml of fresh TPGY broth in duplicate and incubated at 12°C or 30°C. One ml of each mid-exponential-phase culture was sampled and diluted (1:1) with 5% glutaraldehyde (Sigma-Aldrich) for 2 h at room temperature to fix the cells. The samples were washed with sterile water, and 3.5-μl amounts were incubated on carbon-coated grids (Electron Microscopy Sciences, Hatfield, PA) for 2 min. The liquid was carefully removed. Cells were stained by adding 3.5 μl phosphotungstic acid (1%) on the grid. Negative stain was incubated for 15 s and carefully removed. The grids were analyzed with the Tecnai 12 transmission electron microscope (Philips Electron Optics, Holland) in the electron microscopy unit of the Institute of Biotechnology, University of Helsinki.

RESULTS

Expression of *clo3403* and *clo3404* is induced after a cold shock. The expression of the CLO3403/CLO3404 TCS in the cold shock response of *C. botulinum* E1 Beluga was studied by measuring the relative mRNA levels of *clo3403* and *clo3404* immediately before (t_0) and 1 min, 30 min, 1 h, 2 h, 5 h, and 24 h after a cold shock (temperature downshift from 30°C to 12°C) using RT-qPCR. A nonshocked culture served as a control (Fig. 1). The cold shock induced the expression of the histidine kinase (*clo3403*) and response regulator (*clo3404*) genes 1.5- to 3.4-fold in the first 5 h relative to their expression at time point t_0 (Fig. 2A). At 24 h after the cold shock, the expression of both genes (*clo3403* and *clo3404*) was significantly downregulated relative to their expression at t_0 ($P < 0.05$; the expression ratios were 0.16 and 0.11, respectively). The nonshocked control culture showed downregulation of the TCS genes already at 2 h and 5 h (expression ratios of 0.37 to 0.04) (Fig. 2B). Calibrating the results for the cold-shocked culture to the results for the nonshocked culture revealed more than 10-fold (not significant) and more than 100-fold ($P < 0.05$) induction of *clo3403* and *clo3404* mRNA expression in the cold-shocked culture at 2 h and 5 h, respectively (Fig. 2C).

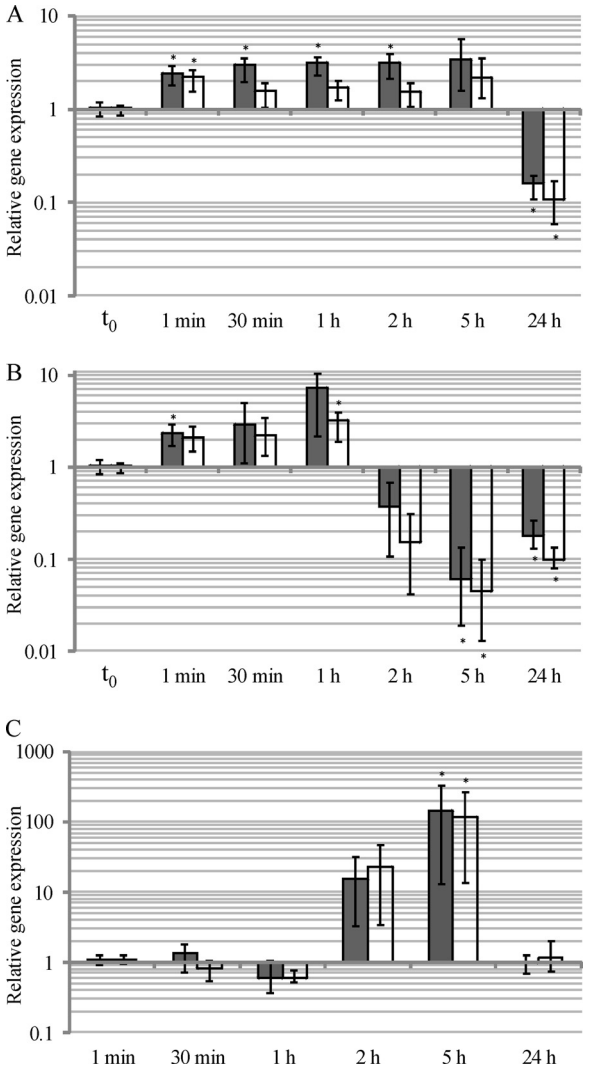

FIG 2 Expression of *clo3403* (dark gray) and *clo3404* (white) is induced after a cold shock. Relative expression levels of *clo3403* and *clo3404* in cold-shocked culture calibrated to t_0 (A), in nonshocked culture calibrated to t_0 (B), and in cold-shocked culture calibrated to nonshocked culture at corresponding time points (C). Gene expression was normalized to 16S *rrn*. Error bars indicate the minimum and maximum values of three biological replicates. *, $P < 0.05$.

Insertional inactivation of *clo3403* and *clo3404*. To examine the role of the CLO3403/CLO3404 TCS in growth at low temperature (12°C), insertional knockout mutants were constructed. The genes encoding *clo3403* and *clo3404* were disrupted by inserting a mobile group II intron from *Lactococcus lactis* (Ll.*ltr*B) using the ClosTron system. Insertion in the correct site was confirmed by PCR using primers flanking the intron insertion site (Fig. 3A). The resulting $clo3403_s$ (sense) and $clo3403_a$ (antisense) and $clo3404_s$ and $clo3404_a$ mutants were confirmed by amplifying a 2,615-bp and a 2,311-bp fragment, respectively. Correct orientation of the insertion was analyzed by PCR using a gene-specific primer and the intron-binding EBS Universal primer (Fig. 3B). The $clo3403_s$ and $clo3403_a$ mutants were confirmed by obtaining a 679-bp and an 837-bp fragment, respectively, and the $clo3404_s$ and $clo3404_a$ mutants by obtaining a 365-bp and a 323-bp fragment, respectively. Single intron insertion was shown by Southern blot-

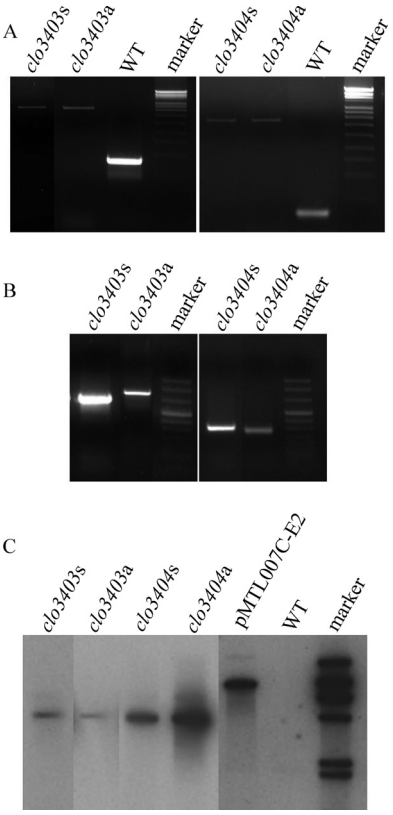

FIG 3 Insertional inactivation of *clo3403* and *clo3404* in sense (s) and antisense (a) orientation using the ClosTron. (A and B) Insertion in the correct site was confirmed by PCR using primers flanking the intron insertion site (A), and insertion in correct orientation was confirmed by PCR using a gene-specific primer and the intron-binding EBS Universal primer (B). (C) Southern blot analysis of HindIII-digested genomic DNA from the WT and *clo3403* and *clo3404* mutants using an intron-specific probe.

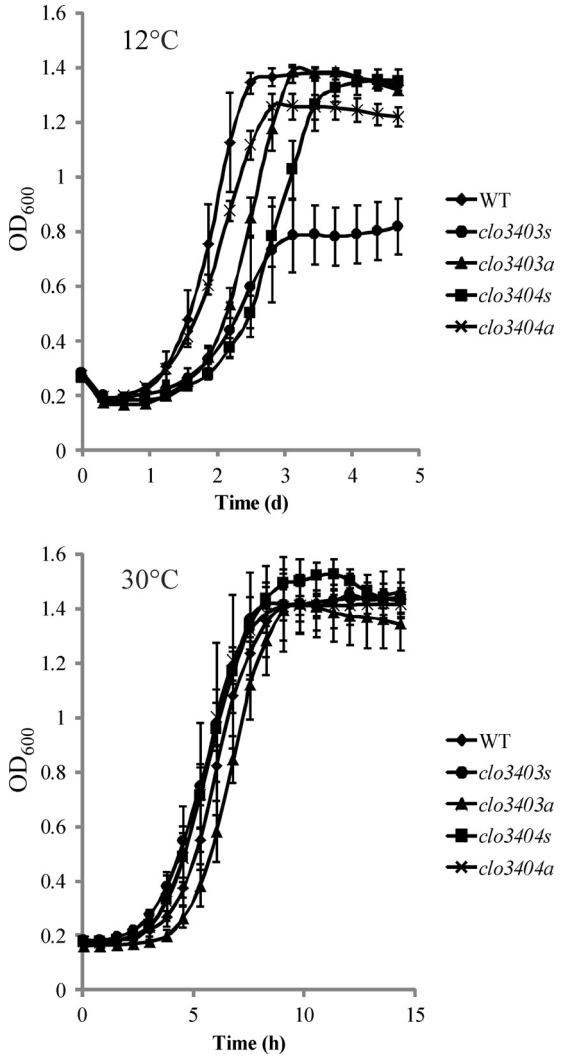

FIG 4 The *clo3403* and *clo3404* mutants show impaired growth at 12°C. Growth curves of WT and *clo3403*$_s$, *clo3403*$_a$, *clo3404*$_s$, and *clo3404*$_a$ strains at 12°C and 30°C. Volumes of 350 μl of diluted ONCs (1:100 diluted into fresh TPGY broth) were incubated at 12°C or 30°C in the Bioscreen C microbiology reader in an anaerobic workstation. OD_{600} values were measured automatically at 15-min intervals. Error bars represent standard deviations of three biological replicates.

ting using an intron-specific probe (Fig. 3C). HindIII-digested genomic DNA of each mutant gave one band on the Southern blot. Digested WT DNA was the negative control. To our knowledge, the *clo3403* and *clo3404* mutants constructed are the first confirmed and reported insertional knockout mutants of group II *C. botulinum*.

The *clo3403* and *clo3404* mutants showed impaired growth at 12°C. The TCS mutants showed impaired growth at 12°C compared to the growth of the WT strain (Fig. 4). Both *clo3403* mutants exhibited an extended lag phase, and the *clo3403*$_s$ mutant was unable to reach the WT level of cell density. The *clo3404*$_s$ mutant showed a prolonged lag phase but reached the WT level of cell density, whereas the growth of the *clo3404*$_a$ mutant was slightly impaired compared to that of the WT, with a delayed (~8 h) entry into stationary growth phase and slightly reduced maximum cell density. Determining the maximum growth rates revealed significantly ($P < 0.01$) reduced maximum growth rates of all four TCS mutants relative to the growth of the WT at 12°C (Table 3). These negative effects on growth were not observed in cultures growing at the optimum temperature (30°C). Additionally, the role of the CLO3403/CLO3404 TCS in growth at low temperature was confirmed by successful complementation of the mutations (Fig. 5). The mutants harboring pMTL82151::*clo3404-clo3401* showed improved growth (increased maximum cell densities and increased maximum growth rates) at 12°C compared to the growth of the vector control strains, and their maximum growth rates were fully restored to WT levels. The results suggest that the functional CLO3403/CLO3404 TCS is needed for efficient growth at low temperature.

The *clo3403*$_s$ mutant is not motile. The motility of *C. botulinum* E1 Beluga and the TCS mutants was studied by stab inoculation into TPGY agar (0.3%) and culture at 12°C and 30°C. The WT, *clo3404*$_s$, and *clo3404*$_a$ strains showed clear motility at 12°C and 30°C after 2 days of incubation. The *clo3403*$_s$ mutant was not motile at all at either temperature, and *clo3403*$_a$ showed reduced motility at 12°C compared to that of the WT (Fig. 6). Hence, *C. botulinum* E1 Beluga is motile at optimum and low temperature, whereas inactivation of *clo3403* has a negative effect on motility. Inactivation of *clo3404* does not affect motility.

The motility of the *clo3403*$_s$ mutant was fully restored at 12°C

TABLE 3 Maximum growth rates of WT, complementation, and vector control strains at optimum and/or low temperature[a]

Strain description	Mean maximum growth rate (OD_{600}/h) ± SD at:	
	12°C	30°C
WT	0.045 ± 0.000	0.313 ± 0.028
clo3403$_s$	0.019 ± 0.009[b]	0.279 ± 0.001
clo3403$_a$	0.041 ± 0.001[b]	0.315 ± 0.013
clo3404$_s$	0.033 ± 0.003[b]	0.341 ± 0.029
clo3404$_a$	0.033 ± 0.004[b]	0.322 ± 0.017
WT-pMTL82151	0.026 ± 0.005	
WT-pMTL82151::*clo3404-clo3401*	0.026 ± 0.001	
clo3403$_s$-pMTL82151	0.021 ± 0.006	
clo3403$_s$-pMTL82151::*clo3404-clo3401*	0.028 ± 0.009	
clo3403$_a$-pMTL82151	0.005 ± 0.002	
clo3403$_a$-pMTL82151::*clo3404-clo3401*	0.032 ± 0.001[c]	
clo3404$_s$-pMTL82151	0.008 ± 0.004	
clo3404$_s$-pMTL82151::*clo3404-clo3401*	0.026 ± 0.003[c]	
clo3404$_a$-pMTL82151	0.018 ± 0.000	
clo3404$_a$-pMTL82151::*clo3404-clo3401*	0.024 ± 0.002[c]	

[a] WT, *clo3403*$_s$, *clo3403*$_a$, *clo3404*$_s$, *clo3404*$_a$, and respective complementation and vector control strains were grown at 12°C and/or 30°C.
[b] Result is significantly ($P < 0.01$) different from the value for the WT.
[c] Value is significantly ($P < 0.01$) increased compared to the value for the respective vector control.

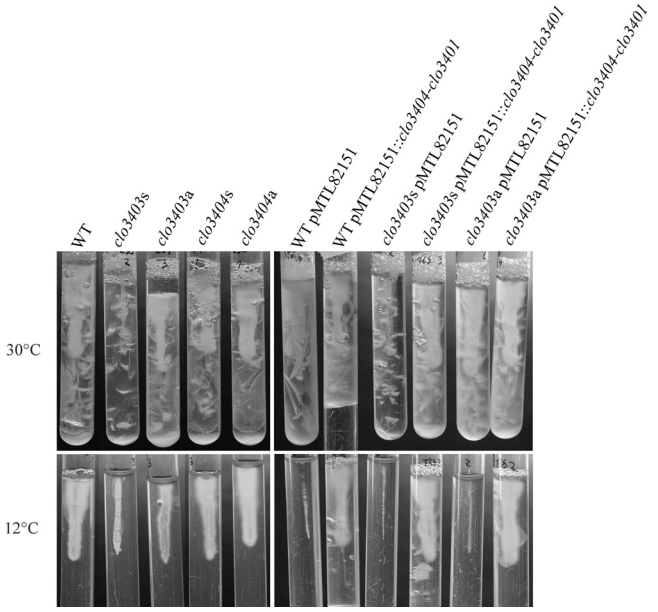

FIG 6 The *clo3403*$_s$ mutant is nonmotile, and its motility is restored by introducing pMTL82151::*clo3404-clo3401*. Motility assays of WT, *clo3403*$_s$, *clo3403*$_a$, *clo3404*$_s$, and *clo3404*$_a$ (left) strains at 12°C and 30°C and of WT, *clo3403*$_s$, and *clo3403*$_a$ strains harboring pMTL82151 or pMTL82151::*clo3404-clo3401* (right) at 12°C and 30°C. Single colonies were stab inoculated into tubes containing TPGY agar (0.3%) and incubated at 12°C or 30°C. Images were taken after 2 days of incubation, with the exception of the image showing complementation at 12°C taken after 4 days of incubation.

and 30°C by introducing the complementation plasmid pMTL82151::*clo3404-clo3401* (Fig. 6), and the motility of the *clo3403*$_a$ mutant at 12°C was restored by introducing pMTL82151::*clo3404-clo3401*. Surprisingly, the WT carrying the empty vector control was nonmotile at 12°C, but this could be an effect of antibiotic pressure and/or plasmid replication effort under cold stress, as the WT was shown to be motile in the absence of the antibiotic and the vector. Nevertheless, all strains carrying the complementation plasmid were fully motile at both temperatures, affirming the possible role of the CLO3403/CLO3404 TCS, or at least the histidine kinase CLO3403, in the motility of *C. botulinum* E1 Beluga.

The *clo3403*$_s$ mutant does not form flagella. To study whether disturbed flagellum synthesis could explain the nonmotility of the *clo3403*$_s$ mutant, we performed electron microscopy analysis. The *clo3403*$_s$ mutant did not form flagella during mid-exponential growth phase at 12°C and 30°C, whereas the WT formed flagella at both temperatures (Fig. 7). Similar to the WT, the *clo3404*$_s$, *clo3404*$_a$, and *clo3403*$_a$ mutants were motile and produced flagella at 12°C and 30°C (data not shown). Thus, the nonmotile phenotype of *clo3403*$_s$ is most probably linked to its missing flagella. The

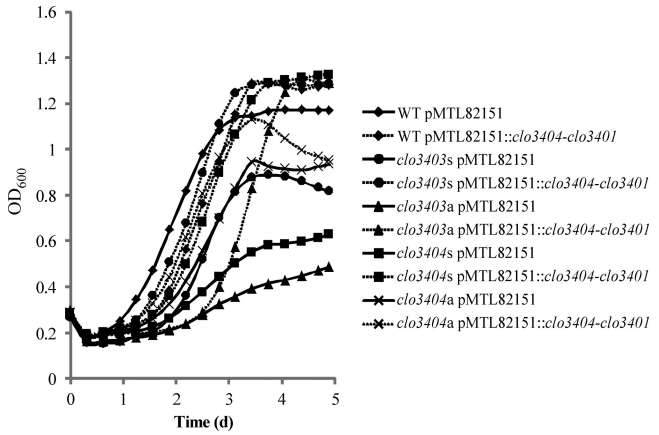

FIG 5 Improved growth of TCS mutants at 12°C by introducing pMTL82151::*clo3404-clo3401*. Growth curves of WT, *clo3403*$_s$, *clo3403*$_a$, *clo3404*$_s$, and *clo3404*$_a$ strains harboring the empty vector control (pMTL82151) or the complementation plasmid (pMTL82151::*clo3404-clo3401*) at 12°C. Volumes of 350 μl of diluted ONCs (1:100 diluted into fresh TPGY broth) were incubated at 12°C in the Bioscreen C microbiology reader in an anaerobic workstation. OD_{600} values were measured automatically at 15-min intervals. Curves represent the means of three biological replicates.

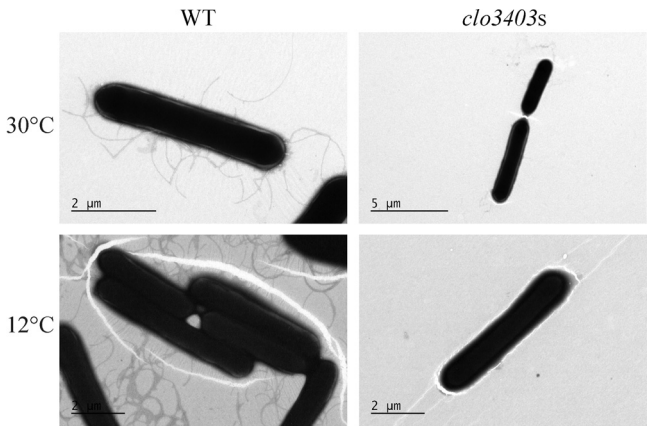

FIG 7 The *clo3403*$_s$ mutant does not form flagella. Electron microscopy images of WT and *clo3403*$_s$ strains. The *C. botulinum* ONCs were diluted (1:100) into 50 ml of fresh TPGY broth in duplicate and incubated at 12°C or 30°C. Samples for transmission electron microscopy were taken during mid-exponential growth phase at 12°C and 30°C.

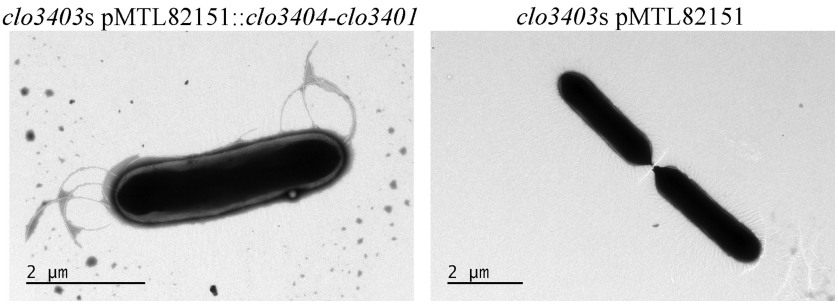

FIG 8 The flagellum formation of the *clo3403*$_s$ mutant is restored by introducing pMTL82151::*clo3404-clo3401*. Electron microscopy images of the *clo3403*$_s$ mutant harboring pMTL82151::*clo3404-clo3401* and the *clo3403*$_s$ mutant harboring pMTL82151. The *C. botulinum* ONCs were diluted (1:100) into 50 ml of fresh TPGY broth and incubated at 30°C. Samples for transmission electron microscopy were taken during mid-exponential growth phase.

complemented *clo3403*$_s$ mutant was motile and produced flagella (Fig. 8).

Electron microscopy analysis also revealed that the cell size (3 to 5 μm) and cell shape are very similar among the tested strains during mid-exponential growth phase at both temperatures, confirming that the differential optical densities measured between the WT and mutant cultures at 12°C were growth related and not caused by varying cell sizes.

DISCUSSION

Determining the relative expression levels of the TCS genes *clo3403* and *clo3404* revealed induction of expression for at least 5 h after a cold shock, whereas in the nonshocked culture grown at the optimum temperature (30°C), the relative mRNA levels of *clo3403* and *clo3404* were downregulated at 2 h and 5 h. The prolonged induction of the TCS genes after the cold shock is suggested to be a response to the temperature downshift. TCSs responding to low temperature on the transcriptional level have been reported in group I *C. botulinum* organisms (27, 28). Similar to the current study, the previous works showed that the relative expression levels of *cbo0366*/*cbo0365* and *cbo2306*/*cbo2307* in group I *C. botulinum* organisms was most increased 5 h after the cold shock when calibrating the cold-shocked culture to the nonshocked culture. Thus, we suggest that TCSs in group I and group II *C. botulinum* are responding to low temperature in a similar time range. In *Yersinia pseudotuberculosis*, 44 TCS genes were significantly induced under cold stress (23). It is thus plausible that *C. botulinum* is also using multiple TCSs to respond to cold. At 24 h after the cold shock, the expression of the TCS genes *clo3403* and *clo3404* was downregulated to similar levels in cold-shocked and nonshocked cultures, which is most likely a growth-phase-dependent effect.

The involvement of the CLO3403/CLO3404 TCS in growth at low temperature was demonstrated by impaired growth (significantly reduced maximum growth rates) of the *clo3403*$_s$, *clo3403*$_a$, *clo3404*$_s$, and *clo3404*$_a$ mutants at 12°C compared to the WT growth. At the optimum growth temperature, no growth difference was observed between the mutants and the WT. Comparison between the mutants with sense and antisense mutations of *clo3403* or *clo3404* showed that the insertion orientation had an effect on the intensity of the cold-sensitive phenotype and motility in the *clo3403* mutants. As discussed earlier, these differences may be linked to polar effects of the strong *erm* promoter within the intron or, more likely, to the presence of antisense RNA (28). Insertion of the intron in the sense orientation within *clo3403* could lead to antisense RNA production against the upstream regulator gene, *clo3404*, thus not only disrupting *clo3403* but also attenuating *clo3404* and thereby knocking out the entire TCS. We hypothesize that the more-drastic phenotypes observed for *clo3403*$_s$ than for *clo3403*$_a$ were due to such a total silencing of this TCS. A disturbed flagellum synthesis most probably affected the cold tolerance of this mutant. Another explanation for the phenotypic differences between the sense and antisense mutants could be the different intron insertion sites used for constructing the mutants. In the *clo3403*$_s$ mutant, the insertion site was in the C-terminal HAMP domain, whereas the antisense mutant had the insertion in the N-terminal sensing domain. Therefore, insertion site-dependent functional differences cannot be excluded. Nevertheless, all of the mutants grew less efficiently than the WT at 12°C, and this finding was further confirmed by successful complementation of the mutations. Loss of growth efficiency at low temperature (15°C) was also reported for the group I *C. botulinum* TCS mutants with mutations of *cbo0366*, *cbo0365*, *cbo2306*, and *cbo2307* (27, 28), which affirms the general role of TCSs in the cold tolerance of *C. botulinum*. A protein blast analysis suggests that neither CBO0366/CBO0365 nor CBO2306/CBO2307 is the closest homologue to CLO3403/CLO3404. The closest homologous proteins to CLO3403/CLO3404 are CLH0370/CLH0369 and CLLA0382/CLLA0381 in *C. botulinum* Alaska E43 and *C. botulinum* Eklund 17B, respectively. Whether those TCSs are also important for the cold tolerance of *C. botulinum* Alaska E43 and *C. botulinum* Eklund 17B must be tested. More research is needed to solve the mechanism of TCSs behind the cold adaptation of this dangerous pathogen.

The *clo3403*$_s$ histidine kinase mutant, the most cold-sensitive mutant among those tested, appeared nonmotile and nonflagellated, and the *clo3403*$_a$ mutant showed reduced motility at 12°C. In contrast, the *clo3404*$_s$ and *clo3404*$_a$ mutants were fully motile, which suggests that the response regulator CLO3404 alone is not essential for motility or that other regulators may compensate for it. As discussed above, the nonmotile phenotype of the *clo3403*$_s$ mutant could be explained by a combined effect of the disrupted *clo3403* and the antisense RNA-attenuated *clo3404*, silencing the entire TCS. How the blocking of this TCS affects flagellation remains to be characterized. The operon encoding most of the flagellar genes is located 400 kb downstream from the TCS genes, making polar effects on motility highly unlikely. The motility of *clo3403*$_s$ and *clo3403*$_a$, however, was fully restored by introducing the complementation plasmid, which affirms the role of the histi-

dine kinase in motility. Furthermore, motility seems to be important for the growth of *C. botulinum* E1 Beluga at the low but not at the optimum temperature. This result is consistent with previous hypotheses that motility and cold growth are linked in bacteria (23, 50, 51). In *Y. pseudotuberculosis*, the CheA/CheY TCS was shown to regulate motility and to be involved in the cold stress response. *cheA* and *cheY* were highly induced at 3°C, and a functional *cheA* was important for growth at cold temperature (23). Similar findings were made in *Listeria monocytogenes*, in which the response regulators DegU and GmaR played a role in the temperature-dependent regulation of flagellar genes (52–54) and the flagellar proteins FlhA and MotA were needed for efficient growth at low temperature (50). Moreover, in *E. coli*, the flagellar genes *fliC*, *fliH*, and *fliN* were highly induced after a temperature downshift (55).

In conclusion, we demonstrate that the CLO3403/CLO3404 TCS of the psychrotrophic food-borne pathogen *C. botulinum* type E is required for efficient growth at low temperature and that a cold shock induces the expression of the TCS for at least 5 h. We also showed that inactivation of the histidine kinase gene *clo3403* has a negative effect on motility. The results were confirmed by successful complementation of the mutations.

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