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The two-component system CBO2306/CBO2307 is important for cold adaptation of *Clostridium botulinum* ATCC 3502

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ABSTRACT

Clostridium botulinum is a notorious foodborne pathogen. Its ability to adapt to and grow at low temperatures is of interest for food safety. Two-component systems (TCSs) have been reported to be involved in cold-shock and growth at low temperatures. Here we show the importance of TCS CBO2306/CBO2307 in the cold-shock response of *C. botulinum* ATCC 3502. The relative expression levels of the *cbo2306* and *cbo2307* were up to 4.4-fold induced in the cold-shocked cultures but negatively regulated in the late-log and stationary growth phase in relation to early logarithmic growth phase in non-shocked cultures. Importance of the CBO2306/CBO2307 in the cold stress was further demonstrated by impaired growth of insertional *cbo2306* or *cbo2307* knockout mutants in relation to the wild-type strain ATCC 3502. The results suggest that the TCS CBO2306/CBO2307 is important for cold-shock response and adaptation of *C. botulinum* ATCC 3502 to low temperature.

1. Introduction

Strains of Group I *Clostridium botulinum* are notorious foodborne pathogens that are widespread in nature. Proteolytic Group I strains form highly heat-resistant spores, hence they may survive food processing (Lindström and Korkeala, 2006). In contaminated foods, spores may germinate and outgrow. During its vegetative growth, *C. botulinum* produces highly potent botulinum neurotoxin. Therefore, it is imperative to control the growth of *C. botulinum* in foods.

Thermal control is commonly used to prevent the growth of pathogenic bacteria in the modern foods, thus temperature abuse may have dangerous consequences (Lindström et al., 2006, 2010; Peck, 2006). In recent years an increasing research interest has been focused on the genetic mechanisms behind bacterial responses to thermal stress.

Cold stress has been studied in the model organism *Bacillus subtilis* (Aguilar et al., 1998, 1999; Beranová et al., 2007, 2010; Brigulla et al., 2003; Budde et al., 2006; Graumann et al., 1996; Wiegeshoff et al., 2006). One of its well-known cold-induced systems is the two-component system (TCS) DesK/DesR. Upon activation, the O_2-dependent *des*-encoded Δ5-desaturase increases the amount of unsaturated fatty acids in the cell membrane, resulting in increased membrane fluidity (Aguilar et al., 1998, 1999, 2001; Beranová et al., 2010). TCSs are specialized signal transduction systems responding to diverse environmental stimuli including pH, osmolarity, oxidative stress, and temperature. TCSs generally consist of a membrane associated sensor histidine kinase and a cognate response regulator. The histidine kinase senses the environmental signal and undergoes ATP-dependent autophosphorylation at a conserved histidine residue. Subsequently, its phosphoryl group is transferred to the cognate response regulator which results in phosphorylation of an aspartate residue in the response regulator. The DNA-binding activity of the response regulator then mediates specific changes in gene expression (Dutta et al., 1999; Hoch, 2000; Mitrophanov and Groisman, 2008).

The involvement of the TCS CBO0366/CBO0365 in cold adaptation of Group I *C. botulinum* ATCC 3502 was recently shown (Lindström et al., 2012). The role of the other TCSs has not been studied in the cold-shock response of *C. botulinum*. Here we show that the TCS CBO2306/CBO2307 is important in the cold-shock response and adaptation of *C. botulinum* ATCC 3502 at low temperature.

2. Materials and methods

2.1. Strains, culture and sampling

The Group I *C. botulinum* ATCC3502 parent and *cbo2306* or *cbo2307* mutant strains were streaked on blood agar plates (5% of bovine blood per liter of agar) and incubated for 2 days at 37 °C. Three single colonies of each strain were separately inoculated into 10 ml aliquots of tryptone–peptone–glucose–yeast-extract (TPGY) broth (50 g/l of tryptone, 5 g/l peptone, 20 g/l of yeast extract [Difco Becton Dickinson, Sparks, MD], 4 g/l of glucose, [VWR International, Leuven, Belgium], and 1 g/l of sodium thioglycolate [Merck KGaA, Darmstadt, Germany]) and

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incubated at 37 °C for 24 h. A volume of 100 μl of each culture was further inoculated into 10 ml of fresh TPGY broth and incubated at 37 °C. After 16 h of incubation, all cultures were diluted to an optical density (OD_{600}) of 1.0 with fresh TPGY for further studies.

All cultures were confirmed to carry the botulinum neurotoxin gene by multiplex polymerase chain reaction (PCR) (Lindström et al., 2001). All cultures were prepared in an anaerobic work station (MK III, Don Whitley Scientific, Ltd., Shipley, UK) under atmospheric conditions of 85% N_2, 10% CO_2, and 5% H_2. All agar plates and broth were deoxygenated before use by anaerobic storage for 48 h or boiling for 15 min, respectively.

To observe the relative expression of *cbo2306* and *cbo2307* in the *C. botulinum* ATCC 3502 during optimal growth and after cold-shock, 4-ml aliquots of cultures grown for 16 h were inoculated into 400 ml of fresh TPGY broth. The cultures were grown anaerobically at 37 °C until they reached the mid-exponential growth phase, and calibrator samples (t_0) were collected. Immediately after t_0, one half of the culture was cold-shocked in an ice-water bath by letting the culture temperature decrease from 37 to 15 °C, followed by incubation at 15 °C after the cold-shock. The other half of the culture (the non-shocked control culture) was incubated at 37 °C.

Sampling was done simultaneously for the cold-shocked and the non-shocked cultures immediately (1 min) after the cold-shock (t_1), 30 min (t_2), 2 h (t_3), and 5 h (t_4) after cold-shock (Fig. 1). Five-milliliter samples were transferred into 15-ml Falcon tubes containing 1 ml of chilled stop solution (900 μl of 99.6% ethanol [Altia Corporation Rajamäki, Finland] 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 5000 ×*g* at 4 °C. The supernatant was removed, and the cell pellets were stored at −70 °C until RNA purification.

2.2. RNA isolation and reverse transcription

The frozen cell pellets were resuspended in 0.25 ml of lysis buffer containing 25 mg/ml lysozyme (Sigma-Aldrich, St. Louis, MO, USA) and 250 IU/ml mutanolysin (Sigma-Aldrich) in Tris–EDTA buffer (pH 8.0; Fluka Biochemica, Buchs, 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. For reverse transcription (RT), 800 ng of total RNA was reverse transcribed into cDNA using the DyNAmo cDNA Synthesis Kit (Finnzymes, Espoo, Finland) according to the manufacturer's instructions. The RT reaction was carried out in duplicate for each RNA sample. A minus-RT control, with RT enzyme replaced by 2 μl of water, was performed to control DNA contamination.

2.3. Relative gene expression analysis of cbo2306 and cbo2307

The relative gene expression experiments and data analysis were performed as described by Selby et al. (2011). The DyNAmo Flash SYBR Green quantitative PCR (qPCR) chemistry (Finnzymes) was used for qPCR performed in the 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). The experiment was performed in triplicate. The 16S rRNA was used as a normalization reference and the relative expression levels were calculated using the method described by Pfaffl (2001). Primers used in the study are presented in Table 1.

2.4. Construction of cbo2306 and cbo2307 knockout mutants

For construction of the *cbo2306* and *cbo2307* insertional knockout mutants from the ATCC 3502 parent, the ClosTron system was applied (Heap et al., 2010). The insertions were targeted to *cbo2306* in sense orientation at position 879–880 (*cbo2306*s) and in antisense orientation at position 114–115 (*cbo2306*a). Similarly, in *cbo2307* the insertion positions were 150–151 (*cbo2307*s) and 283–284 (*cbo2307*a), respectively. The retargeting plasmids pMTL007C-E2::*cbo2306*-879s, pMTL007C-E2::*cbo2306*-114a, pMTL007C-E2::*cbo2307*-150s, and pMTL007C-E2::*cbo2307*-283a were obtained from DNA 2.0 Inc. (Menlo Park, CA, USA). The vectors were transferred to the ATCC 3502 recipient by conjugation from the donor *Escherichia coli* CA434 strain (Purdy et al., 2002) and transconjugants were selected using cycloserine (250 μg/ml) and thiamphenicol (15 μg/ml). The integrants were confirmed for loss of the pMTL007C-E2 retargeting plasmid by plating on TPGY with thiamphenicol (15 μg/ml) selection.

The successful disruption of *cbo2306* and *cbo2307* was checked with primers flanking the insertion site (Table 1). The insert specific EBS Universal Primer and the gene specific forward or reverse flanking primer were used to check the correct sense or antisense orientation of the insertion, respectively. To confirm a single insertion event into the chromosome, Southern blotting analysis was used as described previously (Cooksley et al., 2010; Heap et al., 2007, 2010; Palonen et al., 2011). Primers and probes used in the study are presented in Table 1.

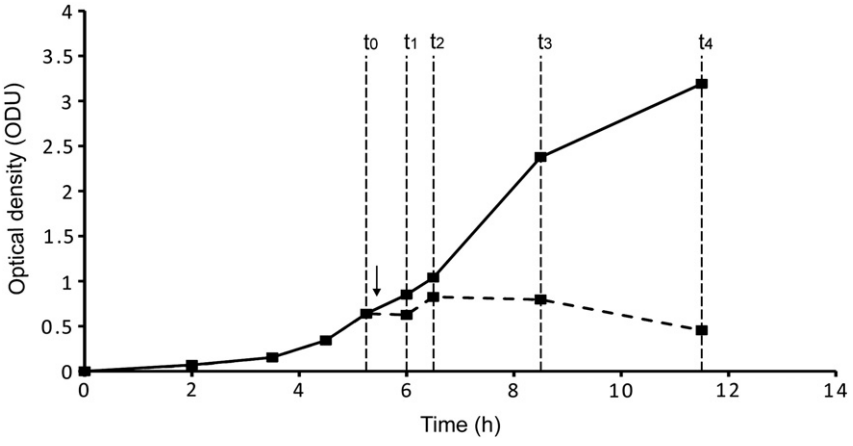

Fig. 1. *Clostridium botulinum* ATCC 3502 was grown at 37 °C, exposed to a temperature downshift to 15 °C at logarithmic growth phase (OD_{600} of 0.8 to 1.0), and incubated at 15 °C for 5 h (dashed growth curve). The arrow indicates the time of cold-shock. Samples were taken for qRT-PCR analysis before cold-shock (t_0) and 1 min (t_1), 30 min (t_2), 2 h (t_3), and 5 h (t_4) after cold-shock. A non-shocked culture (continuous growth curve) served as a control.