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Involvement of Two-Component System CBO0366/CBO0365 in the Cold Shock Response and Growth of Group I (Proteolytic) *Clostridium botulinum* ATCC 3502 at Low Temperatures

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The role of the two-component system (TCS) CBO0366/CBO0365 in the cold shock response and growth of the mesophilic *Clostridium botulinum* ATCC 3502 at 15°C was demonstrated by induced expression of the TCS genes upon cold shock and impaired growth of the TCS mutants at 15°C.

High and low temperatures are used to control the growth and toxigenesis of harmful bacteria in foods. While high temperatures are prone to kill the bacteria, low temperatures often retard bacterial growth without killing them. Bacteria have developed strategies to sense and adapt to low temperatures. While the cellular mechanisms explaining cold shock tolerance and growth of the model organisms *Escherichia coli* and *Bacillus subtilis* at the lower end of their growth temperature ranges have been widely explored (1–6, 8–11, 16, 17, 26, 31, 32, 38), there are scarce reports on such mechanisms for the notorious food pathogen *Clostridium botulinum* (36).

Two-component signal transduction systems are central to bacterial sensing and adaptation to environmental changes (25, 27, 30). The two-component system (TCS) histidine kinase senses environmental stimuli with a sensor domain in its N terminus and sends the signal, through autophosphorylation of a histidine residue in its C-terminal transmitter domain, to the TCS response regulator. An aspartate residue in the receiver domain of the response regulator further transmits the phosphoryl group to the C-terminal output domain of the response regulator. Response regulators possess DNA-binding activity, ultimately resulting in a specific response in target gene expression. TCSs in bacteria are differentially specialized to respond to a wide variety of chemical and physical stimuli, including pH, osmolarity, oxidative stress, and temperature. TCSs associated with a response to low temperature in other bacteria include the DesK/DesR in *B. subtilis* (1–3, 6), CheA/CheY in *Yersinia pseudotuberculosis* (31), CorS/CorR in *Pseudomonas syringae* (37), and Fp1516/Fp1517 in *Flavobacterium psychrophilum* (23). In addition, the LisK/LisR, Lmo1173/Lmo1172, and Lmo1061/Lmo1060 systems were linked to the cold shock response but not to long-term growth of *Listeria monocytogenes* at low temperature (12). The role(s) of TCSs in the cold shock response or adaptation of *C. botulinum* to low growth temperatures is unknown. Here we show that the TCS CBO0366/CBO0365 (39) is involved with the cold shock response and growth of the model strain *C. botulinum* ATCC 3502 at 15°C, a temperature close to this strain's minimum growth temperature (24).

To study the involvement of the TCS CBO0366/CBO0365 in the cold shock response, the relative mRNA levels of *cbo0365* and *cbo0366* in ATCC 3502 cultures (Table 1) were measured via quantitative reverse transcription-PCR(qRT-PCR) (31, 35) immediately before (T0) and 1 min, 30 min, 2 h, and 5 h after a

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TABLE 1 Bacterial strains and plasmids

Strain or plasmid	Relevant properties	Source[a] (reference)
Bacterial strains		
C. botulinum ATCC 3502	Wild type	ATCC (34)
C. botulinum ATCC 3502 *cbo0365*::intron-*erm*	Insertion deletion in *cbo0365*	This study
C. botulinum ATCC 3502 *cbo0366*::intron-*erm*	Insertion deletion in *cbo0366*	This study
E. coli TOP10	Electrocompetence	Invitrogen, Paisley, UK
E. coli CA434	Conjugation donor	UNOTT (33)
Plasmids		
pMTL82153	pBP1 g-positive replicon, *catP*, ColE1 g-negative replicon, *tra*, *fdx* promoter	UNOTT (22)
pMTL82153-*cbo0366*	pMTL82153 with *cbo0366* under transcriptional control of *fdx* promoter	This study
pMTL007	ClosTron plasmid, *catP*, intron with *ermB* RAM	UNOTT (21)
pMTL007-*cbo0365*-48s	Derived from pMTL007 by retargeting to *cbo0365*	This study
pMTL007-*cbo0366*-267s	Derived from pMTL007 by retargeting to *cbo0366*	This study

[a] ATCC, American Type Culture Collection; UNOTT, University of Nottingham, United Kingdom.

TABLE 2 Oligonucleotide primers

Primer	Sequence (5′→3′)[a]	Use[c]	Binding site in ATCC 3502 genome[b]
cbo0365-f	AATGATGCCTAAGATGGATGGT	qRT-PCR	424216–424237
cbo0365-r	TCTGCACCTGTGGTTAATCCT	qRT-PCR	424324–424344c
cbo0366-f	GGCATACCAAGAGCAGAAACCA	qRT-PCR	425289–425310
cbo0366-r	ATTTGCAGCAAGCCCTTTGA	qRT-PCR	425421–425440c
16S *rrn*-f	TTGTCGTCAGCTCGTGTCGT	qRT-PCR	10290–10309
16S *rrn*-r	CCTGGACATAAGGGGCATGA	qRT-PCR	10431–10450c
cbo0366-f-NdeI	NNNNNN<u>CATATG</u>AAACCTTTCAAATATATA	Construction of overexpression plasmid for *cbo0366*	424779–424796
cbo0366-r-NheI	NNNNNN<u>GCTAGC</u>TTATTCATCCTCTGCCATAA	Construction of overexpression plasmid for *cbo0366*	426235–426254c
EBS Universal	CGAAATTAGAAACTTGCGTTCAGTAAAC	Retargeting of pMTL007, control for correct mutation site in *cbo0365* and *cbo0366*	NA
cbo0365-IBS	AAAAAAGCTTATAATTATCCTTAAAAGACATCAGAGTG CGCCCAGATAGGGTG	Construction of pMTL007-*cbo0365*-48s	NA
cbo0365-EBS1d	CAGATTGTACAAATGTGGTGATAACAGATAAGTCATC AGAGATAACTTACCTTTCTTTGT	Construction of pMTL007-*cbo0365*-48s	NA
cbo0365-EBS2	TGAACGCAAGTTTCTAATTTCGGTTTCTTTCCGATAG AGGAAAGTGTCT	Construction of pMTL007-*cbo0365*-48s	NA
*cbo0365*M-f	TTGTTGATGATGAAAAAGAAATCA	Control of pMTL007-*cbo0365*-48s	424074–424097
cbo0366-IBS	AAAAAAGCTTATAATTATCCTTAACTATCCAAGAAGT GCGCCCAGATAGGGTG	Construction of pMTL007-*cbo0366*-267s	NA
cbo0366-EBS1d	CAGATTGTACAAATGTGGTGATAACAGATAAGTCCAA GAACTTAACTTACCTTTCTTTGT	Construction of pMTL007-*cbo0366*-267s	NA
cbo0366-EBS2	TGAACGCAAGTTTCTAATTTCGATTATAGTTCGATAG AGGAAAGTGTCT	Construction of pMTL007-*cbo0366*-267s	NA
*cbo0366*M-f	TTTTTAATGGCTATTATGACCTTTATT	Control of pMTL007-*cbo0366*-267s	424866–424892

[a] Underlined portions of sequences indicate the restriction endonuclease site.
[b] Based on EMBL accession number AM412317 (positions are shown according to base pair number). c, complement strand; NA, not applicable.
[c] 48s and 267s represent mutations between bases 48 and 49 and between bases 267 and 268, respectively, in the sense orientation.

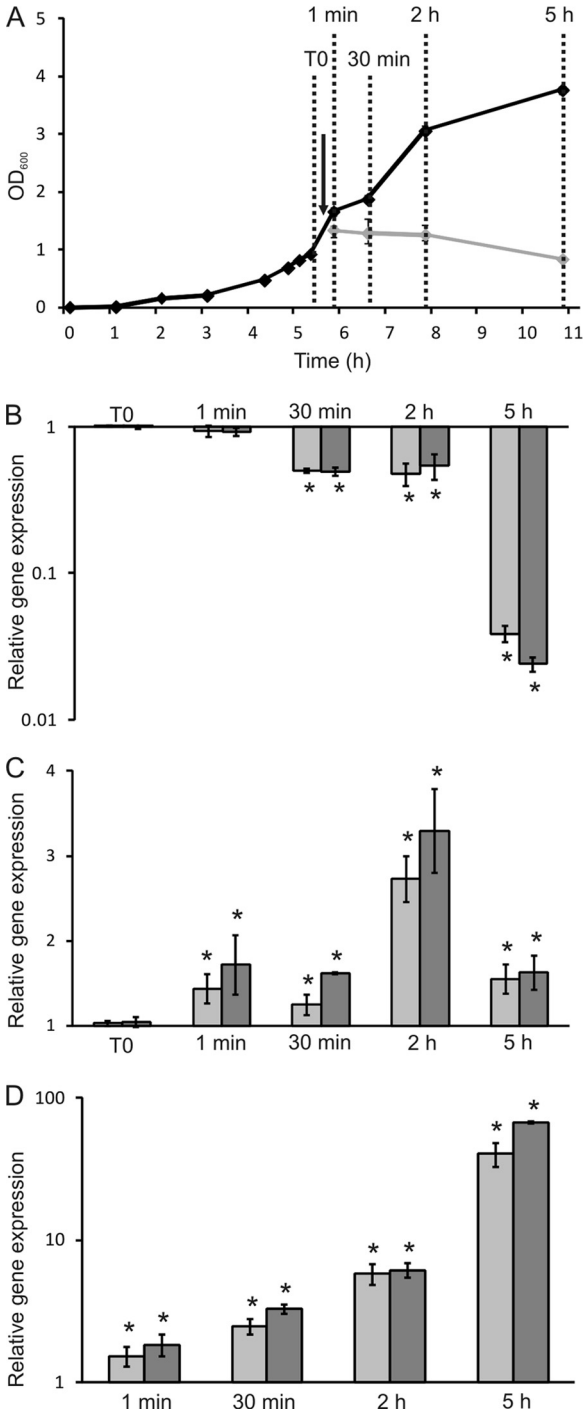

FIG 1 Relative expression levels of *cbo0365* and *cbo0366* in ATCC 3502 induced at 15°C. (A) ATCC 3502 was grown at 37°C, exposed to a temperature downshift (cold shock [gray curve]) at 15°C at an optical density at 600 nm (OD_{600}) of 1.5, and sampled for qRT-PCR analysis before cold shock (T0) and 1 min, 30 min, 2 h, and 5 h after cold shock. A non-cold-shocked culture (black curve) served as a control. (B) Relative expression levels of *cbo0365* (light gray) and *cbo0366* (dark gray) in non-cold-shocked cultures calibrated at T0. (C) Relative expression levels of *cbo0365* (light gray) and *cbo0366* (dark gray) in cold-shocked cultures calibrated at T0. (D) Relative expression levels of *cbo0365* (light gray) and *cbo0366* (dark gray) in cold-shocked cultures calibrated to non-cold-shocked cultures at the corresponding time points. The normalization reference was 16S *rrn*. *, $P < 0.05$ (one-way analysis of variance). Error bars indicate standard deviations of three replicates.

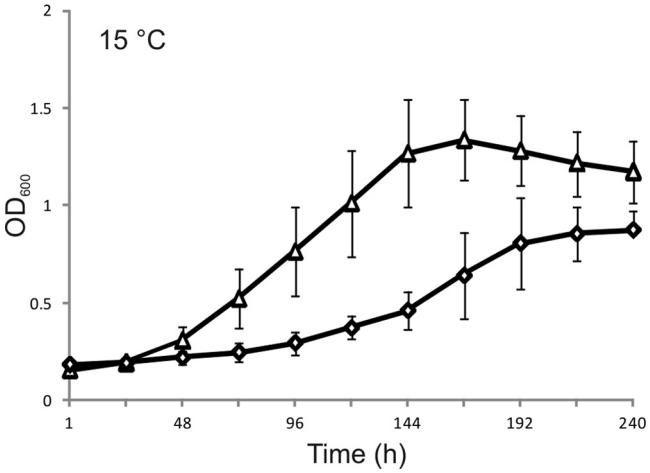

FIG 2 Overexpression of *cbo0366* improves growth of ATCC 3502 at cold temperatures. ATCC 3502 harboring pMTL82153-*cbo0366* (white triangles) or empty pMTL82153 (white diamonds) was grown in tryptose-peptone-glucose-yeast extract (TPGY) medium at 15°C. Error bars indicate standard deviations of three replicates.

temperature downshift from 37°C to 15°C with the primers presented in Table 2. A non-cold-shocked culture served as a control (Fig. 1A). In the non-cold-shocked culture at 37°C, the relative mRNA levels of *cbo0365* and *cbo0366* were significantly downregulated (expression ratios, 0.2 to 0.02; $P < 0.05$) in late-log and stationary growth phases in relation to the early logarithmic phase (T0) (Fig. 1B). In the cold-shocked culture, however, the relative mRNA levels of *cbo0365* and *cbo0366* were 1.2- to 3.3-fold induced ($P < 0.05$) at all time points in relation to T0 (Fig. 1C), suggesting that the gene upregulation was specifically linked to the cold shock response instead of being a stationary-phase event. When calibrated to the non-cold-shocked cultures, up to 40- and 67-fold higher relative *cbo0365* and *cbo0366* transcript levels, respectively, were measured in the cold-shocked culture (Fig. 1D). These results suggest that a cold shock response and the immediate acclimation of ATCC 3502 to low temperature, as depicted by the growth lag of the cold-shocked culture (Fig. 1A), involve induced expression of the TCS genes *cbo0365* and *cbo0366*. While constitutive expression and delicately balanced control through a phosphorelay system have been reported for most TCSs under normal growth conditions (18, 19, 24, 30), induced TCS expression under cold stress conditions has been reported for *B. subtilis* and the psychrotrophic *Y. pseudotuberculosis* (4, 31).

The role of induced *cbo0366* expression in the growth of ATCC 3502 at 15°C was further confirmed by introducing pMTL82153-*cbo0366*, containing the coding sequence of *cbo0366* under transcriptional control of the *fdx* promoter (22), into ATCC 3502 and comparing its ability to grow at 15°C to that of a control strain carrying pMTL82153. The strain harboring pMTL82153-*cbo0366* showed enhanced growth over the strain with the empty vector (Fig. 2), suggesting that *fdx*-mediated overexpression of *cbo0366* may have assisted ATCC 3502 to grow at cold temperature.

To study the role of intact CBO0366/CBO0365 in adapted growth of ATCC 3502 at low growth temperatures, we constructed *cbo0365* or *cbo0366* single insertional knockout mutants (the strains and plasmids used are presented in Table 1, and the primers are listed in Table 2) as described previously (13, 20–22, 35, 36) and compared the abilities of these mutants to grow at

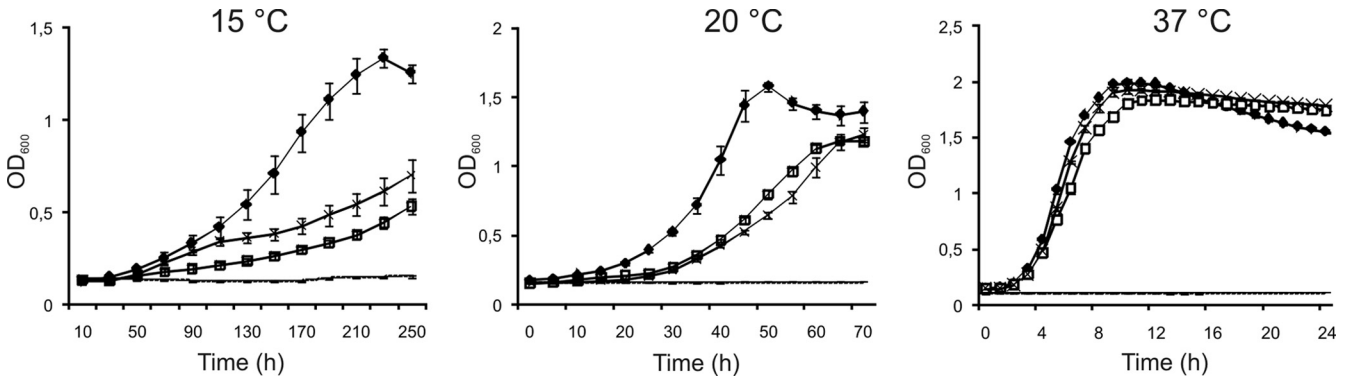

FIG 3 Growth of *cbo0365* and *cbo0366* mutants under cold temperatures is impaired compared to ATCC 3502. ATCC 3502 wild type (black diamonds) and *cbo0365* (open squares) and *cbo0366* (multiplication symbols) mutants were grown in tryptose-peptone-glucose-yeast extract (TPGY) medium at 15°C, 20°C, and 37°C. Results for a negative-control sample (fresh TPGY) are marked with a dashed line. Error bars indicate standard deviations of three replicates.

37°C, 20°C, and 15°C to the wild-type ATCC 3502 as described previously (24, 35) with 24-hour, 3-day, and 11-day follow-up periods. Both *cbo0365* and *cbo0366* knockout mutants exhibited markedly impaired growth at 15°C and 20°C in relation to the wild-type culture. At 37°C, all strains showed similar growth curves (Fig. 3). The results suggest that a functional CBO0366/CBO0365 is required for efficient growth of ATCC 3502 at low temperature but not at its optimum temperature. Our previous report on the role of the cold shock protein-encoding genes, showing a cold-sensitive phenotype for a *cspB* mutant but not for a *cspA* mutant (36), verified that the mutation procedure itself is not responsible for the cold-sensitive phenotype.

As with many cold-responsive TCSs reported (7, 12, 23, 31), the stimulus sensed by the CBO0366 kinase remains to be characterized. The cold-responsive DesK of *B. subtilis* and Hik33 of *Synechocystis* sp. have been shown to respond to cell membrane fluidity and thickness (1–3, 6, 14, 28) and to control desaturation of the fatty acid chains in cell membrane phospholipids, maintaining elasticity of cell membranes in the cold (2, 28). Temperature-dependent alteration of the cell membrane fatty acid composition has been reported for the psychrotrophic group II *C. botulinum* (15), but its regulation is unknown.

While the results demonstrate that the cold shock response and acclimation of ATCC 3502 at low temperature involves induced expression of the CBO0366/CBO0365 genes and that the CBO0366/CBO0365 system is required for efficient growth at 15°C, future work is required to unravel the function of this TCS and the role of its downstream events in the cold tolerance of ATCC 3502.

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