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Role of *csp* genes in NaCl, pH, and ethanol stress response and motility in *Clostridium botulinum* ATCC 3502

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ABSTRACT

Clostridium botulinum is a notable food pathogen and responsible for botulism due to production of botulinum neurotoxin. Strains of *C. botulinum* can adapt to and survive in stress conditions and food processing. The cold shock protein coding genes (*csp*) are involved in growth at low temperature, but they may also possess other functions. In this mutational analysis we show that *cspB* and *cspC*, but not *cspA*, are important for NaCl, pH and ethanol stress responses and for motility of *C. botulinum* ATCC 3502. In all NaCl concentrations tested, the *cspB* mutant had lower maximum growth rate and, together with the *cspC* mutant, a longer lag phase compared to the wild-type strain. At low pH, the *cspB* and *cspC* mutants showed either lower maximum growth rates or longer lag phases compared to the wild type. In all ethanol concentrations tested, the *cspB* mutant had lower maximum growth rates and the *cspC* mutant had a longer lag phase than the wild-type strain. Motility was reduced in *cspA* and *cspC* mutants, and flagella formation was affected. The results suggest that *cspB* plays a universal role in stress response and *cspC* aids *C. botulinum* in NaCl, pH and ethanol stress in *C. botulinum* ATCC 3502.

1. Introduction

Clostridium botulinum is a noted food pathogen. The ubiquitous presence of *C. botulinum* spores in terrestrial and aquatic environments and the high heat-resistance of *C. botulinum* spores pose a contamination risk for raw food materials. *C. botulinum* may survive food processing and multiply and produce neurotoxins in contaminated foods. The consequences of neurotoxin production in foods could be life threatening (Browning et al., 2011; Centers for Disease Control and Prevention, 1998; Forman et al., 2011; Jalava et al., 2011; Lindström and Korkeala, 2006; Lindström et al., 2010).

Conditions such as 10% added NaCl, pH of 4.6, 5.5% (wt/wt) ethanol, or temperatures below 10 or over 48 °C inhibit the growth of Group I *C. botulinum* (Daifas et al., 2003; Hinderink et al., 2009; Peck and Stringer, 2005; Smelt et al., 1982; Smith and Sugiyama, 1988; Stringer et al., 2005). However, such conditions are rarely achieved in the modern foods due to consumer preferences towards natural and minimally preserved foods. Decreasing the factors inhibiting the growth of *C. botulinum* can lead to adaptation and subsequent growth of *C. botulinum*.

Low temperature is commonly used to inhibit the growth of pathogenic bacteria. Cold stress induces the production of cold shock proteins (Csps) found in many prokaryotes and eukaryotes (Brandi et al., 1994; Graumann and Marahiel, 1998; Mihailovich et al., 2010; Wistow, 1990). Three Csps designated as *cspA* (*cbo0282*), *cspB* (*cbo1387*), and *cspC* (*cbo1772*) have been identified in *C. botulinum* ATCC 3502 (Sebaihia et al., 2007) (These genes are not to be mixed with those encoding serine proteases with same designated names [*cspA*, *cspB*, and cspC] that differ in size and function (Adams et al., 2013; Shimamoto et al., 2001)). The relative expression of all three *csp* genes was significantly induced upon cold-shock (Söderholm et al., 2011) and *cspB* was shown to encode the major Csp involved in cold shock response (Söderholm et al., 2011). Csps are not only important for cold stress but also for other cellular processes (Brandi et al., 1994; Graumann and Marahiel, 1999; Wilkinson and Shyu, 2001) and stress conditions (Bae et al., 1999; Loepfe et al., 2010; Phadtare and Inouye, 2001). No reports are available on the role of *csp* genes in non-cold related stress in *C. botulinum*.

Knowing the stress adaptation and related mechanisms in *C. botulinum* may reveal novel biomarkers for growth and thus help to control the growth of *C. botulinum* in foods. In this study, we showed an insertional mutation in the *cspB* or *cspC*, but not in *cspA*,

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hampers the growth of *C. botulinum* ATCC 3502 in NaCl, pH, and ethanol stress. Flagella formation and motility were affected by mutation of the *csp* genes.

2. Materials and methods

2.1. Strains and culture

The Group I *C. botulinum* ATCC 3502 parent and *cspA*, *cspB*, and *cspC* insertional knock-out mutants (Heap et al., 2007; Söderholm et al., 2011) were used in the study. The insertion site for *cspA*, *cspB*, and *cspC* were base 58 (anti-sense orientation), 74 (anti-sense), and 54 (sense), respectively (Söderholm et al., 2011). The ATCC 3502 wild-type strain and the *csp* mutants were streaked on blood agar plates (5% of bovine blood per liter of agar) and incubated for 2 days at 37 °C. A single colony of each strain was separately inoculated into 10 ml aliquots of tryptone–peptone–glucose–yeast-extract (TPGY) broth (50 g/l of tryptone, 5 g/l peptone, 4 g/l of glucose, 20 g/l of yeast extract, and 1 g/l of sodium thioglycollate; Difco, Becton Dickinson, Sparks, MD) and incubated at 37 °C for 24 h. A volume of 100 μl of each culture was further inoculated into 10 ml aliquots of fresh TPGY broth and incubated at 37 °C for 16 h. The overnight cultures, confirmed to carry the botulinum neurotoxin gene by multiplex PCR (De Medici et al., 2009; Lindström et al., 2001), were diluted to optical density (OD_{600}) of 1.0 with fresh TPGY. All cultures were prepared in an anaerobic workstation (MK III, Don Whitley Scientific, Ltd., Shipley, UK) under atmospheric conditions of 85% N_2, 10% CO_2, and 5% H_2. Agar plates and broth used in the study were deoxygenated before use by anaerobic storage for 48 h or 15 min of boiling, respectively.

2.2. Growth characteristics of the ATCC 3502 wild-type and csp mutant strains in NaCl, pH and ethanol stress

A volume of 100 μl of each dilution was inoculated into 10 ml of TPGY broth with 1.0, 1.5, 2.0, 2.5, 3.0, 3.5 or 4.0% (wt/vol) NaCl; buffered TPGY with pH adjusted to 5.5, 6.0, 6.5, 7.0, 7.5 or 8.0; or TPGY with 1.0, 2.0, 3.0, 4.0, 5 and 6% (wt/wt) ethanol (Altia corporation, Rajamäki, Finland). A 350-μl volume of each culture was pipetted in two technical replicate wells of a 100-well microtiter plate and incubated at 37 °C in a Bioscreen C Microbiology Reader (Oy Growth Curves AB, Helsinki, Finland) placed in an anaerobic workstation (MK III, Don Whitley Scientific, Ltd., Shipley, UK) (Derman et al., 2011). The cultures were grown under continuous shaking for 48 h. The OD_{600} values of the cultures were measured automatically and reported at 15-min intervals by the Bioscreen Reader. The experiments were repeated three times. Growth curves of the cultures were obtained by plotting optical density against time. The maximum growth rate (ODU per hour) and the lag phase (h) of each culture was determined by fitting the growth curves to the Baranyi and Roberts's model (Baranyi and Roberts, 1994) by using the DMfit Microsoft Excel program (Institute of Food Research, Norwich, UK).

2.3. Motility assay

The mutants and the wild-type strain were stab-inoculated into TPGY agar tubes containing 0.3% agar. Tubes were incubated at 37 and 20 °C for 24 h and 1 week, respectively.

2.4. Electron microscopy

Bacterial cultures of the wild-type strain and the mutant strains were grown in TPGY broth to their early logarithmic growth phase at 20 or 37 °C. One millliliter of each culture was mixed with 1 ml of 5% glutaraldehyde solution (Sigma–Aldrich, St. Louis, MO) and incubated for 2 h at room temperature. Cells were washed with sterile water, and a drop of bacterial suspension was placed on a carbon-coated grid and the preparation was stained in 3% phosphotungstic acid for 15 s. Grids were examined with the FEI Tecnai 12 transmission electron microscope (Philips Electron Optics, Holland).

2.5. Statistical analysis

The significance of differences in maximum growth rates and lag phases of growth were compared between each mutant and the wild-type strain in different stress conditions by using Student's *t*-test.

Table 1
Mean growth characteristics of *Clostridium botulinum* ATCC 3502 and its *cspA*, *cspB*, *cspC* mutant strains in different growth conditions at 37 °C.

Condition	ATCC 3502		*cspA* mutant		*cspB* mutant		*cspC* mutant	
	GR[a]	Lag[b]	GR	Lag	GR	Lag	GR	Lag
1.0% NaCl	0.31 ± 0.04	4.21 ± 0.57	0.31 ± 0.02	4.91 ± 0.77	0.27 ± 0.04	6.26 ± 1.31	0.33 ± 0.10	5.97 ± 1.58
1.5% NaCl	0.29 ± 0.03	4.93 ± 0.41	0.30 ± 0.04	4.88 ± 0.56	0.23 ± 0.02	7.60 ± 1.14	0.38 ± 0.03	7.37 ± 0.96
2.0% NaCl	0.24 ± 0.02	6.00 ± 0.27	0.24 ± 0.03	6.22 ± 1.25	0.16 ± 0.00	9.00 ± 1.45	0.33 ± 0.01	8.60 ± 0.75
2.5% NaCl	0.21 ± 0.01	7.89 ± 0.37	0.21 ± 0.02	8.36 ± 1.32	0.15 ± 0.02	12.57 ± 1.10	0.26 ± 0.05	10.40 ± 0.74
3.0% NaCl	0.17 ± 0.01	10.50 ± 0.31	0.21 ± 0.01	13.00 ± 1.91	0.16 ± 0.01	18.93 ± 2.12	0.20 ± 0.03	12.80 ± 1.04
3.5% NaCl	0.13 ± 0.00	13.80 ± 0.81	0.16 ± 0.01	16.83 ± 3.53	0.10 ± 0.01	25.88 ± 1.78	0.19 ± 0.00	16.62 ± 1.06
4.0% NaCl	0.10 ± 0.01	16.24 ± 1.39	0.14 ± 0.00	27.19 ± 0.79	0.06 ± 0.01	30.83 ± 0.68	0.16 ± 0.02	20.57 ± 0.15
pH 5.0	NG[c]	NG	NG	NG	NG	NG	NG	NG
pH 5.5	0.23 ± 0.04	8.36 ± 1.30	0.19 ± 0.01	6.56 ± 0.61	0.22 ± 0.02	12.64 ± 4.15	0.16 ± 0.01	7.43 ± 0.65
pH 6.0	0.26 ± 0.04	4.67 ± 0.28	0.27 ± 0.04	4.65 ± 0.13	0.23 ± 0.02	6.11 ± 1.26	0.24 ± 0.01	4.72 ± 0.35
pH 6.5	0.32 ± 0.02	4.18 ± 0.37	0.41 ± 0.07	3.52 ± 0.62	0.28 ± 0.03	4.41 ± 0.93	0.29 ± 0.03	4.24 ± 0.74
pH 7.0	0.37 ± 0.05	4.30 ± 0.18	0.37 ± 0.05	3.23 ± 0.62	0.36 ± 0.06	4.46 ± 0.88	0.30 ± 0.02	4.37 ± 0.49
pH 7.5	0.33 ± 0.02	4.07 ± 0.32	0.36 ± 0.04	3.23 ± 0.45	0.32 ± 0.024	4.27 ± 1.04	0.31 ± 0.03	4.63 ± 0.34
pH 8.0	0.31 ± 0.02	4.03 ± 0.29	0.35 ± 0.05	3.19 ± 0.62	0.31 ± 0.03	4.20 ± 0.75	0.29 ± 0.05	4.32 ± 0.64
1.0% ethanol	0.27 ± 0.04	2.40 ± 0.15	0.32 ± 0.04	1.90 ± 0.51	0.23 ± 0.02	1.50 ± 0.13	0.22 ± 0.02	2.63 ± 0.41
2.0% ethanol	0.21 ± 0.04	3.29 ± 0.36	0.24 ± 0.02	2.36 ± 0.49	0.14 ± 0.02	2.79 ± 0.71	0.17 ± 0.01	3.46 ± 0.25
3.0% ethanol	0.09 ± 0.03	4.69 ± 0.83	0.20 ± 0.02	3.53 ± 0.63	0.05 ± 0.01	2.65 ± 0.55	0.10 ± 0.02	4.91 ± 0.38
4.0% ethanol	0.03 ± 0.01	NC[d]	0.07 ± 0.03	6.45 ± 1.52	NC	NC	NC	NC
5.0% ethanol	NC	NC	NC	NC	NC	NC	NC	NC

[a] Mean maximum growth rate (ODU/h).
[b] Lag phase (h) ± SD.
[c] No growth observed.
[d] No sufficient growth for calculation.