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Alternative Sigma Factor SigK Has a Role in Stress Tolerance of Group I *Clostridium botulinum* Strain ATCC 3502

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The role of the alternative sigma factor SigK in cold and osmotic stress tolerance of *Clostridium botulinum* ATCC 3502 was demonstrated by induction of *sigK* after temperature downshift and exposure to hyperosmotic conditions and by impaired growth of the *sigK* mutants under the respective conditions.

The ability of the anaerobic, Gram-positive, spore-forming, food-borne pathogen *Clostridium botulinum* to survive, grow, and subsequently produce botulinum neurotoxin in foods (1) raises substantial concern over food safety (2, 3). In minimally processed foods, the extrinsic and intrinsic hurdles used to control the outgrowth and toxin production by *C. botulinum* include refrigeration, heat treatment, increased osmolarity or low water activity, extreme pH, and preservatives (2, 4). Understanding the mechanisms by which food-borne pathogenic bacteria cope with these stress conditions is of key importance in designing modern food safety measures.

Sigma factors are dissociable RNA polymerase subunits that alter the promoter specificity of the RNA polymerase complex under different environmental and growth phase-dependent conditions. A stress-responsive alternative sigma factor, SigB, has been identified (5–8) and has been shown to play a role in cold adaptation of *Listeria monocytogenes* (9) and *Bacillus subtilis* (10). However, the genome of *C. botulinum* ATCC 3502 does not harbor a homolog for *sigB* (11), suggesting mechanisms of general stress response different from those in the Gram-positive model organism *B. subtilis*. A gene encoding a homolog for the sporulation sigma factor SigK of bacilli is present in the genome of *C. botulinum* ATCC 3502 (open reading frame [ORF] CBO2541) (11) and was recently shown to be essential in early stage sporulation in *C. botulinum* (12) and in *Clostridium perfringens* (13, 14) and putatively in transcriptional activation of the sporulation master switch Spo0A of *C. botulinum* (12). In *B. subtilis*, recent findings suggest an interesting interconnection between the decision to sporulate and adaptation to stress as nongrowing "vegetative dormant" cells (15, 16). These observations propose a role for the stress sigma factor SigB in regulating the activity of Spo0A of *B. subtilis*. The lack of *sigB* in the *C. botulinum* ATCC 3502 genome suggests that the network for decision making between sporulation initiation and stress adaptation of this organism is regulated differently from the one proposed for *B. subtilis* (15, 16). We thus sought to investigate the behavior and role of the alternative sigma factor SigK in response to stress in *C. botulinum* ATCC 3502.

The *C. botulinum* ATCC 3502 wild-type strain was evaluated for relative *sigK* expression levels after cold shock, exposure to hyperosmotic conditions, exposure to acidity, or under optimal growth conditions, using quantitative reverse transcription-PCR (primers *sigK*-qPCR-F [5′-ACTTATGGGATGTACTAGGAAGTG-3′] and *sigK*-qPCR-R [5′-TTCTTCTTCATCACTTAGAGGCTTG-3′]) (17–19) and the Pfaffl method (20) for quantitation, with 16S *rrn* expression as a normalization reference (primers 16S*rrn*-qPCR-F [5′-AGCGGTGAAATGCGTAGAGA-3′] and 16S*rrn*-qPCR-R [5′-GGCACAGGGGGAGTTGATAC-3′]). In temperature downshift, cultures grown to the early logarithmic phase at 37°C were rapidly cooled to 15°C and thereafter anaerobically incubated at 15°C for 5 h. The actual temperature of the cultures varied between 14 and 18°C. Modest induction of *sigK* expression was observed immediately (1 min) and 30 min after the cold shock, the expression levels being 1.3-fold ($P < 0.05$) and 1.1-fold (nonsignificant) higher, respectively, than that before shock (Fig. 1). At the later time points, a more marked increase in expression of *sigK* was observed: 2 h after cold shock, the average expression level was 1.4-fold higher and at 5 h the level was 2.8-fold higher than the pre-cold shock expression level (both $P < 0.05$) (Fig. 1). The significant upregulation of *sigK* expression following a temperature downshift, but not at 37°C (Fig. 1), suggests that induction of *sigK* is linked to the cold stress response. Expression of *sigB* in *B. subtilis* has been shown to follow a similar pattern of delayed induction after cold shock, the expression reaching its maximum (i.e., 10-fold higher level than in nonshocked culture) at 4 to 5 h after shock (21).

As for hyperosmotic conditions (exposure to 4.5% [wt/vol] NaCl), a rapid increase in the relative expression level of *sigK* after osmotic shock was observed (Fig. 1): 1.4-fold-induced ($P < 0.05$) and 1.9-fold-induced (insignificant due to large biological variation) expression levels were observed immediately and 15 min after the shock, respectively. Thirty minutes after exposure, *sigK* expression returned to the preexposure level and remained unchanged for 2 h until increasing again by 1.6-fold 5 h after exposure ($P < 0.05$) (Fig. 1). The reinduction of *sigK* at 5 h may suggest a requirement for SigK in growth under hyperosmotic stress. No significant changes in expression of *sigK* were observed after pH downshift (37% HCl) from 6.9 to 5.0 (Fig. 1).

Insertional inactivation mutants of *sigK* with the ClosTron insertion (22, 23) in a sense (*sigK*-427s) or antisense (*sigK*-296as) orientation (12) were evaluated for their growth characteristics at 17 and 37°C under hyperosmotic conditions (4.5% [wt/vol] NaCl) and at pH 6.0, 5.3, and 5.0 (17–19, 24). The growth rate and

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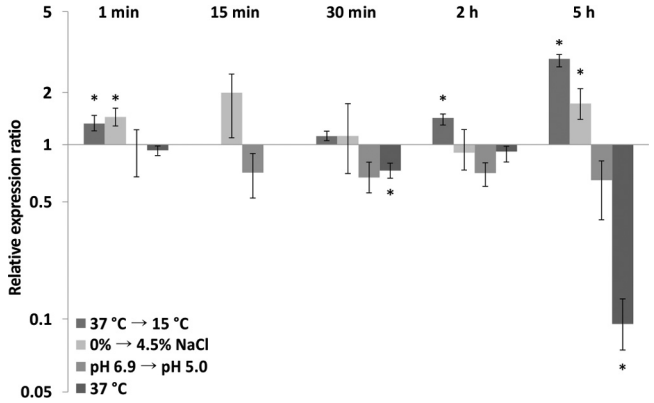

FIG 1 Relative expression of *sigK* in *Clostridium botulinum* ATCC 3502 at given time points after temperature downshift (from 37°C to 15°C), exposure to hyperosmotic conditions (0% to 4.5% NaCl), and pH downshift (from pH 6.9 to pH 5.0), calibrated to expression before exposure in each growth experiment and in cultures grown at 37°C calibrated to expression before temperature downshift. 16S *rrn* expression was used as the normalization reference. The error bars denote the minimum and maximum ratios between three biological replicates. Ratios with a statistically significant difference ($P < 0.05$) are marked with an asterisk.

maximum optical density of the wild-type strain at 17°C were significantly higher than those of the *sigK*-427s and *sigK*-298as mutants (Fig. 2A). The lag times of both mutants determined from the fitted growth curves were slightly decreased compared with that of the wild type (Fig. 2A). At 37°C, the growth rates of all strains were essentially similar (Fig. 2B). The significantly reduced growth rate of both *sigK* mutants at 17°C, compared to the wild-type strain, supports an important role for SigK in adaptation to low temperature. A previous study has shown that ClosTron manipulation (22, 23) does not affect the overall fitness of ATCC 3502 at optimal and cold temperatures (18). The similar behavior of the two *sigK*-427s and *sigK*-298as mutant strains, with intron insertions at different sites and orientations in *sigK*, further supports disruption of *sigK* as the sole cause behind the cold-sensitive phenotype.

In 4.5% NaCl, the growth rates of both mutants were slightly higher than that of the wild type (Fig. 2C). In contrast, the lag phases of both mutants were markedly increased compared with the wild type (Fig. 2C). These data together with the induction of *sigK* after hyperosmotic shock suggest a role for SigK in response and adaptation to hyperosmotic conditions.

At pH 6.0, both mutants reached slightly lower maximum optical densities than the wild-type strain; additionally, the lag phase of the *sigK*-427as mutant was slightly shorter than the lag phase of the wild type (Fig. 2D). However, at pH 5.3, no significant differences in these or other growth characteristics between the mutants and the wild type were observed (Fig. 2D). At pH 5.0, neither the wild type nor the mutants showed any measurable growth (Fig. 2D). As no induction upon pH downshift nor phenotypic differences in growth in culture media buffered to low pH were

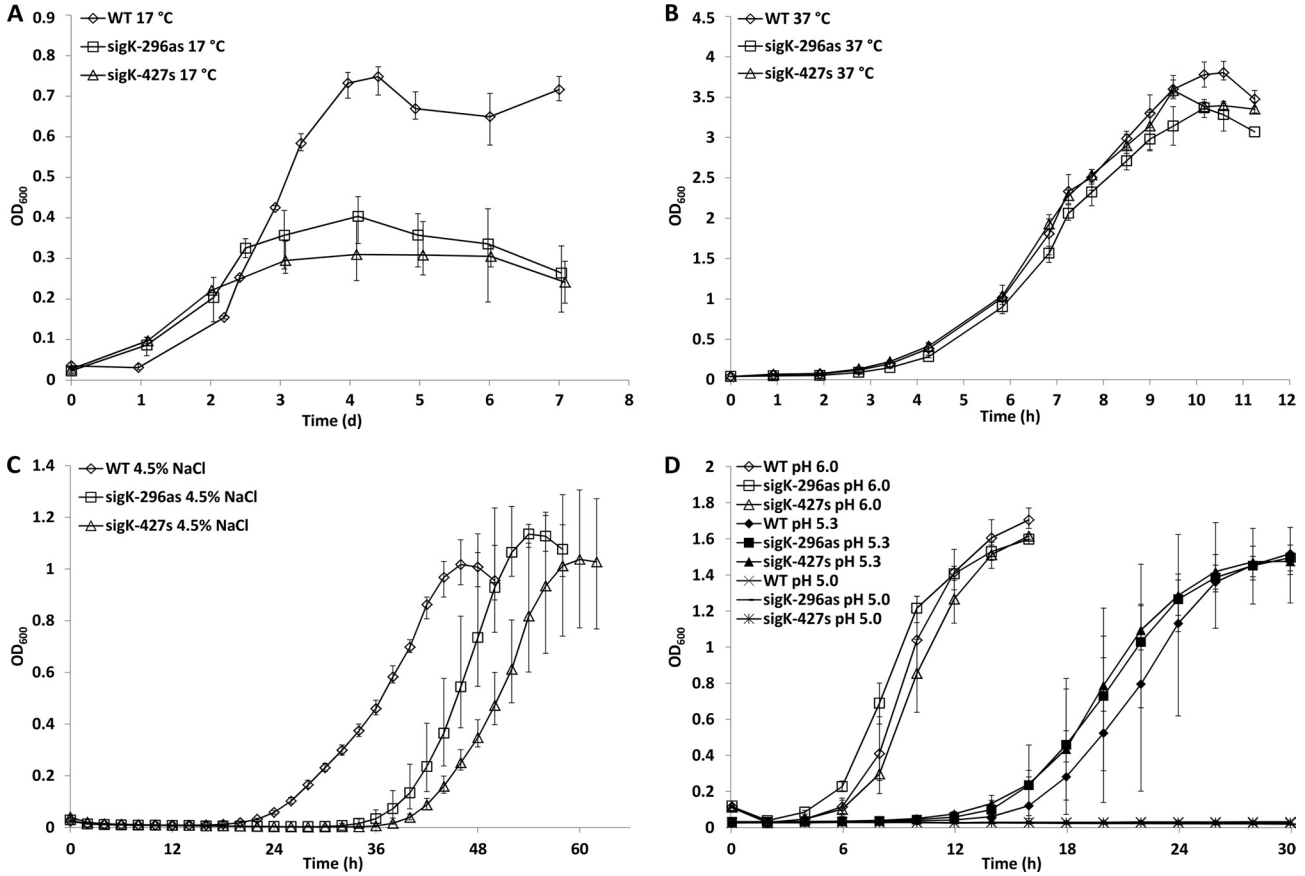

FIG 2 Growth of the *Clostridium botulinum* ATCC 3502 wild-type (WT) strain and *sigK*-296as and *sigK*-427s mutants at 17°C (A) and 37°C (B), in 4.5% (wt/vol) NaCl (C), and at pH 6.0, 5.3, and 5.0 (D). The error bars denote the minimum and maximum optical densities at 600 nm (OD_{600}) of three biological replicates.

observed between the *sigK* mutants and the wild type, it can be concluded that SigK does not have a role in tolerance to acidic conditions.

To date, no information on the role of SigK in stress tolerance of clostridia has been presented. The lack of SigB suggests that different mechanisms are at work in *C. botulinum* to choose between sporulation and other events, such as stress tolerance in a vegetative dormant state as reported for *B. subtilis* (15, 16). Identification of key mechanisms behind response and adaptation to the environmental hurdles *C. botulinum* may encounter in food processing might provide biomarkers to exploit for detection of potentially stress-adapted cells, allowing targeted control methods. Our results suggest a stress-related alternative function for the hitherto strictly sporulation-associated sigma factor SigK of *C. botulinum* ATCC 3502.

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