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Evaluation of normalization reference genes for RT-qPCR analysis of *spo0A* and four sporulation sigma factor genes in *Clostridium botulinum* Group I strain ATCC 3502

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ABSTRACT

Heat-resistant spores of *Clostridium botulinum* can withstand the pasteurization processes in modern food processing. This poses a risk to food safety as spores may germinate into botulinum neurotoxin-producing vegetative cells. Sporulation in *Bacillus subtilis*, the model organism for sporulation, is regulated by the transcription factor Spo0A and four alternative sigma factors, SigF, SigE, SigG, and SigK. While the corresponding regulators are found in available genomes of *C. botulinum*, little is known about their expression. To accurately measure the expression of these genes using quantitative reverse-transcriptase PCR (RT-qPCR) during the exponential and stationary growth phases, a suitable normalization reference gene is required. 16S *rrn*, *adK*, *alaS*, *era*, *gluD*, *gyrA*, *rpoC*, and *rpsJ* were selected as the candidate reference genes. The most stable candidate reference gene was 16S ribosomal RNA gene (*rrn*), based on its low coefficient of variation (1.81%) measured during the 18-h study time. Using 16S *rrn* as the normalization reference gene, the relative expression levels of *spo0A*, *sigF*, *sigE*, *sigG*, and *sigK* were measured over 18 h. The pattern of expression showed *spo0A* expression during the logarithmic growth phase, followed by a drop in expression upon entry to the stationary phase. Expression levels of *sigF*, *sigE*, and *sigG* peaked simultaneously at the end of the exponential growth phase. Peak expression of *sigK* occurred at 18 h, however low levels of expression were detected during the exponential phase. These findings suggest these sigma factors play a role in *C. botulinum* sporulation that is similar, but not equal, to their role in the *B. subtilis* model.

1. Introduction

The formation of heat-resistant spores by the Gram-positive food pathogen *Clostridium botulinum* is a danger to food safety. Pasteurization methods are insufficient in killing Group I *C. botulinum* spores, which may germinate into neurotoxin-producing cultures in improperly stored foods. The molecular mechanisms behind spore formation in *C. botulinum* are poorly understood and much of the current understanding is derived from studies of the Gram-positive spore-forming model organism, *Bacillus subtilis* [1,2].

Homologues of many components of the *B. subtilis* sporulation pathway are found in *C. botulinum*. Both species have the transcriptional master regulator, Spo0A. Four alternative sigma factors, SigF, SigE, SigG, and SigK, govern the spore-formation process in *B. subtilis* and are also found in *C. botulinum* [1,3]. In bacilli, SigF and SigE regulate the early stages of spore formation in the pre-spore and mother cell, respectively, while SigG and SigK regulate the later stages in the pre-spore and mother cell, respectively [4–6]. Recently, similar roles of these regulators have been confirmed in clostridial sporulation, however, some differences to the *Bacillus* model exist [7–12]. In *C. botulinum* ATCC 3502, SigK plays a role in early-stage sporulation [9] and in stress tolerance [13], whereas no such roles have been identified for SigK in *B. subtilis* [14].

Quantitative reverse-transcriptase PCR (RT-qPCR) analysis provides an accurate method for measuring changes in gene expression by quantifying the relative change in the expression of a target gene against a stable normalization reference gene [15,16]. The method requires one or more reference genes that give stable transcripts over the entire study [17]. The expression of ribosomal RNA has been used as an internal control for RT-qPCR in eukaryotes [18], and 16S rRNA (*rrn*) was later adopted as a reference gene in many Gram-positive bacteria such as *Listeria monocytogenes* [19]. Historically, 16S *rrn* has also been used as the normalization

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reference for RT-qPCR in *C. botulinum* [20–27] as transcripts are stable under a variety of conditions. No other potential reference genes have been identified for *C. botulinum*.

In this study, eight reference gene candidates for *C. botulinum* ATCC 3502 (RefSeq accession number NC_009495) were evaluated over the exponential and stationary phases of growth in an 18-h experimental window. Four candidates were selected from the closely related species *Clostridium difficile* [28] and three from a detailed analysis of reference genes in *Acidithiobacillus ferrooxidans* [29]. The selected reference gene candidates were 16S *rrn*, and the genes putatively encoding adenosine kinase (*adK*, RefSeq accession number YP_001255941), alanyl-tRNA synthetase (*alaS*, YP_001255060), GTP-binding protein Era (*era*, YP_001255438), glutamate dehydrogenase (*gluD*, YP_001254311), DNA gyrase subunit A (*gyrA*, YP_001252559), RNA polymerase β′ subunit (*rpoC*, YP_001255969), and 30S ribosomal protein S10 (*rpsJ*, YP_001255963). The candidate reference gene with the most stable transcript was then selected as a normalization reference for analysis of the relative expression of *spo0A* (YP_001254374), *sigF* (YP_001255579), *sigE* (YP_001255031), *sigG* (YP_001255030), and *sigK* (*cbo2541*, YP_001255039) in *C. botulinum* ATCC 3502. Sporulation is asynchronous in *C. botulinum* and other clostridia [30,31]; thus we restricted the transcriptional study to an 18-h time window to monitor the accumulation of sporulation-specific regulatory gene transcripts prior to spore formation in the first cells entering into sporulation.

2. Materials and methods

2.1. Culture conditions

C. botulinum ATCC 3502 was stored as a spore suspension in sterile distilled H_2O at 4 °C. Spores were heated to 80 °C for 5 min prior to inoculation in tryptone–peptone–glucose–yeast extract (TPGY) broth (5% tryptone, 0.5% peptone, 2% yeast extract [Difco, BD Diagnostic Systems, Sparks, MD, USA], 0.4% glucose [VWR International, Leuven, Belgium], 0.1% sodium thioglycolate [Merck, Darmstadt, Germany]) used as a general growth and sporulation medium for *C. botulinum* [9,32,33]. Cultures were incubated at 37 °C in an anaerobic cabinet (MG1000 Anaerobic Work Station; Don Whitley Scientific Ltd., Shipley, UK) in an atmosphere of 85% N_2, 10% CO_2, and 5% H_2. Anaerobic TPGY broth or anaerobic TPGY plates (1.5% agar) were used to grow *C. botulinum* for this experiment.

2.2. Experimental setup

A colony of *C. botulinum* ATCC 3502 on a TPGY plate was inoculated into 10 ml of TPGY broth and grown overnight. One ml from the overnight culture was inoculated into 100 ml of TPGY broth. Optical density (OD_{600} nm) was measured to track growth in each culture. One-ml samples were collected for RNA isolation and subsequent RT-qPCR analysis at six time points representing key growth stages: the exponential phase (5, 7, and 8 h) and stationary phase (10, 15, and 18 h). Samples for RNA isolation were pipetted into 200 μl of ice-cold stop solution containing phenol and ethanol (1:9) and stored on ice for 30 min. The samples were centrifuged at 13,000 rpm for 5 min at 4 °C and the supernatant was discarded. The pellets were stored at −70 °C until RNA isolation. The experiment was performed in triplicate [28].

2.3. RNA isolation, cDNA synthesis and qPCR analysis

Frozen cell pellets were re-suspended in 250 μl of lysis buffer containing lysozyme (50 mg/ml, Sigma Aldrich, St. Louis, MO, USA), mutanolysin (1000 U/ml, Sigma Aldrich) and Tris–EDTA (10 mM Tris–HCl, 1 mM EDTA, pH 8.0), and incubated at 37 °C for 30 min. RNA isolation and purification were performed using the RNeasy Mini Kit (Qiagen, Hilden, Germany) with a DNase treatment (Qiagen). RNA was eluted twice in RNase-free water and a second DNase treatment was performed according to the Ambion DNA-free kit (Applied Biosystems, Life Technologies Corporation, Carlsbad, CA, USA). RNA concentration was measured at 260 nm with the NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). RNA purity was confirmed by measuring the A_{260}/A_{280} ratio with the NanoDrop spectrophotometer and integrity of the RNA was determined using the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). For reverse transcription (RT), a total of 800 ng of RNA was used to synthesize cDNA from random hexamer primers using the DyNAmo cDNA synthesis kit (Thermo Fisher Scientific) as instructed. Duplicate RT reactions (RT replicates) were performed for each RNA sample. RT controls were created from pooled samples of RNA (5 samples per pool) without reverse transcriptase and were included in each run to control DNA contamination of RNA. Controls without template were included in the qPCR runs to show the components of the master mix were not contaminated with DNA. All cDNA samples and RT controls were stored at −20 °C prior to analysis. The Maxima SYBR Green qPCR Master Mix kit (Thermo Fisher Scientific) was used for all qPCR reactions. Each reaction contained 12.5 μl of master mix (Thermo Fisher Scientific), 0.3 μM of each primer, 4 μl of diluted (10^{-6}-fold for 16S *rrn*; 10^{-4}-fold for *adK*, *gluD*, *rpoC*, *rpsJ*, and *sigF*; and 10^{-3}-fold for *alaS*, *era*, *gyrA*, *spo0A*, *sigE*, *sigG*, and *sigK*) cDNA template, and ultrapure water up to a final volume of 25 μl. qPCR runs (Rotor Gene 3000 Real Time Thermal Cycler, Qiagen) consisted of an initial enzyme activation step at 95 °C for 10 min, followed by 40 cycles of denaturation at 95 °C for 15 s and annealing and extension at 60 °C for 60 s. A melt curve analysis was performed by raising the temperature at the end of each run in by 0.5 °C per 5 s from 60 °C to 99 °C. Standard curves were created for each primer set with qPCR, using a dilution series of pooled cDNA from individually synthesized samples as a template. This cDNA pool was independent of the RNA pool used as the RT control. The reaction efficiencies for each primer pair (Table 1) were determined from the slopes of these standard curves using the Rotor Gene software. Two PCR replicates were analyzed for each cDNA replicate.

2.4. Reference gene analysis

Primers (Table 1) were designed for the eight reference gene candidates 16S *rrn*, *adK*, *alaS*, *era*, *gluD*, *gyrA*, *rpoC*, and *rpsJ* using the Primer3 software [34,35]. The expression of each gene was detected at the six time points indicated above using qPCR. The threshold cycle (C_q) values [36] were calculated using the Rotor Gene software from the prepared standard curves for each primer set. The stability of the reference gene candidates was evaluated for each gene by ANOVA and pairwise comparisons of the C_q values over 18 h using the Predictive Analytics SoftWare Statistics 18 (IBM). The coefficient of variation (CV), $CV = \sigma/\mu$ where σ is the standard deviation of the C_q values of a candidate reference gene and μ is the mean C_q value for that the same gene, was determined for each reference gene candidate at both growth phases separately, and for the entire duration of the study, to demonstrate stability of the transcripts [37].

2.5. Relative expression analysis of sporulation genes

Primers (Table 1) for *spo0A*, *sigF*, *sigE*, *sigG*, and *sigK* were designed with Primer3. 16S *rrn* was used as the normalization