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ORIGINAL ARTICLE

Evidence for quorum sensing in *Clostridium botulinum* 56A

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Abstract

Aims: Experiments were designed to detect quorum-sensing signals produced by *Clostridium botulinum*.
Methods and Results: *Clostridium botulinum* 56A cell-free supernatants obtained at the end of lag phase, the mid-exponential phase and early stationary phase of growth were assayed for bioluminescence in the *Vibrio harveyi* quorum-sensing assay system. Twelve and 16-h culture supernatants induced bioluminescence in the auto-inducer 2 (AI-2) but not the auto-inducer 1 (AI-1) assay. Intra-species quorum sensing was also assayed as the ability of the supernatants to promote spore germination and outgrowth in a microtitre plate system. Spore populations exposed to *C. botulinum* supernatant from the end of lag phase became positive for growth sooner than controls.
Conclusions: The influence of cell-free supernatant on ungerminated spores and detection of bioluminescence in the AI-2 assay are evidence for a signalling molecule(s) and provide a first step in characterizing *C. botulinum* quorum sensing.
Significance and Impact of the Study: This study suggests that spores do not behave independently of each other and may explain the inocula size effects observed in challenge studies. Whether AI-2 production in *C. botulinum* serves as an inter-species signal or as a detoxification mechanism remains to be determined.

Introduction

Quorum sensing, the ability of cells to communicate with their peers to control cellular functions has been demonstrated in a wide array of bacteria (Bainton *et al.* 1992; Crockford *et al.* 1995; Withers and Nordstrom 1998; Anonymous 1999; Bassler 1999; Hastings and Greenberg 1999; Augustin *et al.* 2000; Dockery and Keener 2001; Gonzalez *et al.* 2001; Miller and Bassler 2001). The importance of quorum sensing to food microbiology has recently been reviewed (Smith *et al.* 2004). However, little is known about quorum sensing in the foodborne pathogen *Clostridium botulinum*. Indeed, independence and lack of interaction are usually assumed by food microbiologists. Mathematical modelling (Zhao *et al.* 2002) and computer simulation (Zhao *et al.* 2003) have demonstrated that this assumption of independence may not be correct. This study seeks to augment those findings with microbiological evidence.

Vibrio harveyi, one of the most studied quorum-sensing bacteria, has two signalling systems, autoinducer-1 (AI-1) and AI-2 (Bassler *et al.* 1993, 1994). The AI-1 signal, an *N*-acyl homoserine-lactone, is used for intra-species communication. The second system, AI-2 detects inter-species signalling (Bassler *et al.* 1997; Surette *et al.* 1999) and contains a boron atom at the active centre of the molecule (Chen *et al.* 2002). These AI-1 and AI-2 systems are redundant for bioluminescence induction in *V. harveyi*. Wild type and mutated *V. harveyi* strains are used to detect inter- and intra- species signalling (Bassler *et al.* 1993, 1994, 1997; Surette and Bassler 1998, 1999; Bassler 1999; Surette *et al.* 1999; Miller and Bassler 2001). Wild-type strain BB120 produces and detects AI-1 and AI-2. Strains BB152 and BB170 are mutants used to produce

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and detect (respectively) AI-2 (Bassler *et al.* 1994). Strain BB886 is a mutant that specifically detects AI-1 (Bassler *et al.* 1993).

This paper presents evidence that *C. botulinum* cells communicate with each other during germination and growth. *Vibrio harveyi* strains detected the presence of the inter-species autoinducer AI-2 in cell-free supernatants of *C. botulinum* cells in the mid-exponential phase of growth. Cell-free supernatant from the end of lag phase applied to ungerminated spores triggered more rapid germination.

Materials and methods

Preparation of cell-free supernatants

All *C. botulinum* culture manipulations were carried out in an anaerobic chamber (Coy Laboratory Products Inc., Grass Lake, MI, USA). *Clostridium botulinum* 56A (originally obtained from the National Food Processors Association, Washington, DC, USA) spores suspended in sterile distilled water were heat-shocked (80°C, 10 min), inoculated into brain heart infusion (BHI; Difco Laboratories, Detroit, MI, USA) broth (pH 6·5 and 0·5% sodium chloride) to a final concentration of *c.* 10^7 spores per ml and incubated at 30°C for various intervals. The culture was then centrifuged and passed through a 0·22 μm filter (Millipore Corporation, Bedford, MA, USA) to obtain spore and cell-free supernatant.

Influence of cell-free supernatant on ungerminated spores

Microtitre plate assays were used to determine if the addition of cell-free supernatant would affect the germination of spores present at low levels. Ten per cent 8·5 h supernatant was mixed with fresh BHI and then 200 μl was allocated into each of eight wells on a 96-well microtitre plate (Corning Costar Co., Cambridge, MA, USA) as uninoculated controls. The BHI was then inoculated with heat-shocked *C. botulinum* 56A spores to achieve a spore concentration of *c.* 25 spores per ml; 200 μl of the inoculated medium was applied to each of 40 wells. Two-hundred microlitres was also plated on each of ten BHI agar plates to confirm the inoculum concentration. Fresh medium controls (eight uninoculated and 40 inoculated wells) were prepared in a similar manner on the same 96-well microtitre plate. The microtitre wells were then covered with 50 μl of sterile mineral oil (Sigma Chemical Co., St Louis, MO, USA) and sealed with an adhesive film (Rainin Instrument Co. Inc., Woburn, MA, USA). Turbidity in each well was followed by a Multiskan MCC/340 reader (MTX Labsystems Inc., Mclean, VA, USA) maintained at 30 or 22°C inside the anaerobic chamber. Absorbance at 620 nm was monitored every hour until most wells turned turbid. The Gompertz equation was fit to the data and the equation parameters were used to calculate the maximum growth rate and time-to-detection (Zhao *et al.* 2000). The *t*-test was used to check whether there was any significant difference between the mean for these two groups. A *P*-value smaller than 0·05 was considered significant.

Bioluminescence assays for AI-1 and AI-2

Four *V. harveyi* strains (Table 1) were kindly provided by Dr Bonnie Bassler, Princeton University. Cell stocks were prepared in AB medium (Greenberg *et al.* 1979) and stored at −70°C.

A modified BHI medium was used for the bioluminescence test. One-hundred millilitres were prepared in the following manner: 3·7 g BHI broth powder and 1·25 g NaCl were mixed with 90 ml distilled water, autoclaved and cooled completely; 12·3 g $MgSO_4$ were mixed with 100 ml distilled water, autoclaved, cooled completely; finally, 90 ml BHI and 10 ml $MgSO_4$ were mixed with

Table 1 Detection of quorum-sensing molecules in *Clostridium botulinum* and *Vibrio harveyi* supernatants using the *Vibrio harveyi* bioluminescence assay

	Relative bioluminescence		
Supernatant source	Strain BB120 (detects AI-1 and AI-2)	Strain BB170 (detects AI-2)	Strain BB886 (detects AI-1)
Vibrio harveyi			
Strain BB120 (produces AI-1 and 2)	1·0	1·0	1·0
Strain BB152 (produces AI-2)	0·63 ± 0·27	1·59 ± 0·38	0·37 ± 0·14
Uninoculated brain heart infusion	0	0	0
Clostridium botulinum supernatants (h)			
8·5	0	0	0
12	0·13 ± 0·12	0·33 ± 0·15	0
16	0·23 ± 0·11	0·35 ± 0·10	0
20	0	0	0

1 ml filter-sterilized 1 mol l^{-1} potassium phosphate buffer (pH 7·0) and 1 ml filter-sterilized 0·1 mol l^{-1}L-arginine.

The test protocol was adapted from Surette and Bassler (1998). *Vibrio harveyi* stock culture was inoculated at 1% v/v into modified BHI medium (freshly prepared) and shaken at 200 rev min^{-1} at 30°C for 16 h. One millilitre each of BB120 and BB152 was then centrifuged and filtered (0·22 μm) to obtain a cell-free supernatant. Strains BB120, BB170 and BB886 cells were diluted 10 000 times using modified BHI medium. Ten percent of supernatant from BB120, BB152 as positive controls, or from similarly centrifuged and filtered *C. botulinum* culture sampled at different times or uninoculated modified BHI (as a negative control) was mixed with the diluted *V. harveyi* reporter cells. One-hundred microlitres of this mixture was allocated to each of two wells on the microtitre plate and one small glass tube (two inches in length and 1/4 inch in diameter). The microtitre plate was sealed with sterile adhesive film and the small glass tube with parafilm (American National Can, Neenah, WI, USA). Both were then secured on a shaker (200 rev min^{-1}, DS-500 Orbital Shaker; VWR Scientific Products, Willard, OH, USA) maintained at 30°C inside an incubator. Turbidity reading for the microtitre plate (Multiskan MCC/340; MTX Labsystems Inc., Mclean, VA, USA) and bioluminescence reading for the glass tubes (Luminoskan TL Plus Luminometer; Labsystems, Helsinki, Finland) were monitored hourly. Bioluminescence readings per unit of turbidity were calculated at 4 h after inoculation (Surette and Bassler 1998). The adjusted bioluminescence reading for a fresh medium sample (negative control) was taken as zero and that for the wild type *V. harveyi* (BB120, positive control) as 1·0. In each of triplicate experiments, the results for the test samples were normalized to these values. An induction ≥10% of the positive control was scored as positive per the protocol of Surette and Bassler (1998).

Experimental hazards

Clostridium botulinum produces a lethal neurotoxin and is a select agent. All procedures were conducted by immunized personnel working in a Select Agent Laboratory registered with the CDC in compliance with the USA Patriot Act. All protocols were in accordance with CDC and NIH guidelines and were approved by the University Biosafety Committee.

Results

In these studies, supernatants from cultures at the end of lag phase, mid-exponential and beginning of stationary phase were obtained at 8·5, 12 and 20 h respectively. The 8·5-h supernatants were used in experiments to determine the influence of cell-free supernatant on ungerminated spores. Supernatants from all three intervals were used in the assays for AI-1 and AI-2.

Influence of cell-free supernatant on ungerminated spores

Two replicates at 30°C and two at 22°C were performed to test the influence of cell-free supernatant on ungerminated spores. Representative data are shown in Fig. 1. The wells, which contained cell-free supernatant, increased in turbidity at a faster rate than those containing only fresh media. This effect was larger at 22°C than at 30°C (30°C data not shown). The mean values of times to detect turbidity from the media containing cell-free supernatants were always smaller, but not always statistically significant at $P < 0·05$. The portion of wells evidencing growth was always higher for the media containing the cell-free supernatant than the unaugmented media. The mean of the growth rate from the supernatant group was significantly smaller at 30°C, indicating that, once growth had been initiated, fresh medium supported growth better than medium augmented with cell-free supernatant. Growth rates were similar for the two groups at 22°C.

Assays for AI-1 and AI-2 in *Clostridium botulinum* cell-free supernatants

Control experiments using *V. harveyi* strains specific for the production or detection of AI-1 or AI-2 illustrate the utility of the assay system (Table 1). The amount of AI-1 or AI-2 produced by strain BB120 is arbitrarily set at 1·0

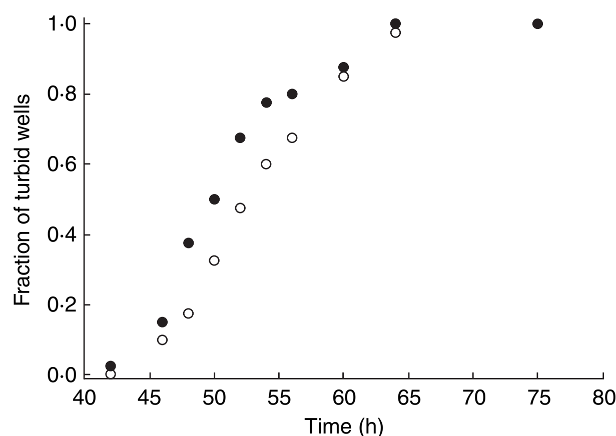

Figure 1 Portion of wells showing growth *vs* time at 30°C for *Clostridium botulinum* spores (*c.* 5 CFU $well^{-1}$) inoculated into fresh media (○) or media augmented with supernatants from *C. botulinum* cultures (●).

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for all reporter strains. Strain BB152, an AI-1$^-$ mutant exhibits decreased bioluminescence in the AI-1 reporter strain, BB886, and increased bioluminescence for the AI-2 specific reporter strain BB170. Uninoculated BHI did not induce bioluminescence in any of the reporter strains.

All of the triplicate cell-free supernatant samples from mid-exponential phase (12–16 h) of *C. botulinum* growth induced bioluminescence in strains BB120 and BB 170, but not in strain BB886, suggesting the presence of AI-2 but absence of AI-1 (Table 1). No induction of bioluminescence occurred with supernatants obtained at the end of lag or the beginning of stationary phase.

Discussion

The mean time-to-detect turbidity from the medium augmented with cell-free supernatants was always smaller than that of unaugmented media. However, statistical tests did not show consistent significance. One possible reason is that the assay system detects time-to-turbidity (of which germination time is a driving step) rather than 'time to germination'*per se*. The time required to detect turbidity is the sum of germination time and the time required for out-growing cell population to double to the detection limit. The detection limit of absorbance measurements is about 10^6 cells per ml. Other methods for detecting germination time, such as release of dipicolinic acid (DPA) and loss of heat-resistance (Dring and Gould 1971), staining using acridine orange (Bruno and Mayo 1995), confocal microscopy (Coote *et al.* 1995) and germination-induced bioluminescence by insertion of the *lux*AB gene from *V. harveyi* (Ciarciaglini *et al.* 2000), were considered. However, all these methods require use of high spore concentrations, making them unsuitable for our studies, which require low spore levels. The use of optimized culture media and conditions or novel high-sensitivity instrumental methods might make the signalling effect from supernatant more pronounced.

Supernatant sampled at mid-exponential phase of *C. botulinum* growth triggered bioluminescence in *V. harveyi* strains BB120 (wild type reporter for AI-1 and AI-2) and BB170 (AI-2 reporter strain), but not in BB886 (AI-1 reporter). This finding is consistent with results indicative of AI-2 production in other organisms (Surette and Bassler 1998, 1999; Surette *et al.* 1999). Our results indicate that a molecule produced during *C. botulinum* germination and growth has a similar chemical composition and conformational structure with the AI-2 signal. Smith *et al.* (2004) note that the role of AI-2, which is being found in an increasing number of organisms, is controversial as it may serve functions other than quorum sensing. Winzer *et al.* (2002) suggest that the widespread microbial production of AI-2 may be a mechanism to detoxify methyl hydroxy-furanone, a by-product of the activated methyl cycle. Methyl hydroxy-furanone is toxic to the cell and can be converted to AI-2, which is excreted (Chen *et al.* 2002). Thus, in some species, AI-2 production may be a mechanism for excreting a toxic substance rather than a means of cellular communication. However, the limited period of signal duration in *C. botulinum* cultures argues against a detoxification role. Fuqua and Greenberg (1998) point out that communication requires not only the sending of a signal, but also receiving and acting on the information. AI-2 certainly meets these criteria in *V. harveyi*, but *V. harveyi*'s ability to receive and respond to AI-2 produced by some other species does not prove that the other species is also receiving and responding to a signal. The target of AI-2 in the organism that produces it should be identified to definitively attribute its role in that organism as quorum sensing. This remains to be performed with *C. botulinum*.

It is not clear whether the molecule (AI-2) responsible for inter-species communication, the molecule used for intra-species communication (AI-1), or some other novel signal triggers faster germination. When sampled at the end of lag phase, the supernatant could trigger faster germination but no bioluminescence. On the contrary, supernatant at the mid-exponential phase could induce bioluminescence, but without stimulating germination. This suggests that the molecule for inter-species signalling in *C. botulinum* is different from that for intra-species signalling.

The influence of cell-free supernatant on ungerminated spores and auto-inducer assays are evidence for a signalling molecule(s) that affect germination and growth of *C. botulinum* spores. The molecule that triggers faster germination may not be the same one detected by the *V. harveyi* bioluminescence assay for AI-2. Whether AI-2 production in *C. botulinum* serves as an inter-species signal or as a detoxification mechanism remains to be determined. This research provides a first step in characterizing quorum sensing in *C. botulinum*.

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