

A Minisonicator To Rapidly Disrupt Bacterial Spores for DNA Analysis

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Concerns about the use of anthrax spores as a weapon of mass destruction have motivated the development of portable instruments capable of detecting and monitoring a suspected release of the agent. Optimal detection of bacterial spores by PCR requires that the spores be disrupted to make the endogenous DNA available for amplification. The entire process of spore lysis, PCR, and detection can take several hours using conventional methods and instruments. In this report, a minisonicator and prototype spore lysis cartridge were built to disrupt *Bacillus* spores in 30 s for rapid, real-time PCR analysis. Utilization of the minisonicator improved PCR analysis by decreasing the limit of detection, reducing the time of detection, and increasing the signal amplitude. Total time of spore disruption and detection using the minisonicator and a microchip PCR instrument was less than 15 min.

Bacillus anthracis (anthrax) spores are recognized as a top candidate for a biological weapon and are often referred to as “the poor man’s atomic bomb”. Spores can be produced in large quantities, stored for decades, and readily disseminated in the air. Establishing a strong defense against anthrax spores requires the development of instrumentation that will provide early warning, identify infected individuals in a timely manner to administer antibiotics, and offer high specificity to distinguish virulent strains from other closely related avirulent forms in order to prevent triggering a false alarm.

Current biochemical and molecular approaches for anthrax spore detection are based on protein and nucleic acid biomarkers. However, since the genetic code is degenerate, tests that target nucleic acids offer higher specificity and precise genetic identification compared to protein substrates. PCR, which was invented over 10 years ago,^{1,2} is an important technique for nucleic acid analysis because of its proven effectiveness, sensitivity, and speed. In addition, new developments in PCR chemistries^{3–7} and

instrumentation^{8–15} have resulted in real-time, homogeneous PCR assays that can be completed in a manner of minutes.

One caveat to PCR analysis of bacterial spores is that optimal detection requires the spores to be disrupted to make the DNA available to the reaction components. However, spores contain an outer spore cortex that is extremely resistant to harsh physical and chemical treatments,¹⁶ making it challenging to identify a spore lysis method that can be completed in less than 1 min, avoids harsh chemicals inhibitory to PCR, and has the potential for integration into an automated, fluidic sample preparation system. In this report, *Bacillus* spores were disrupted and ready for PCR testing in only 30 s using a prototype device consisting of a spore lysis cartridge and minisonicator. This device significantly improved PCR analysis of the spore samples by lowering the limit of detection, reducing the time of detection, and increasing the signal amplitude. The total time required to disrupt the spores using the minisonicator and detect the spores using a microchip PCR instrument was 15 min.

EXPERIMENTAL SECTION

Bacterial Strain and Spore Purification. Dried *B. subtilis* spores were kindly provided by Dugway Proving Grounds, UT.

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Spores were resuspended in water and quantitated by spreading on agar plates containing brain heart infusion (BHI) media (Difco, Mauston, WI). To prepare purified spores, approximately 10^7 of the Dugway spores were germinated in 75 mL of BHI media and incubated overnight at 37 °C in a shaking water bath. A stab of the resultant cells was inoculated into 100 mL of spore media¹⁷ and incubated at 37 °C in a shaking water bath for 3 days. Sporulation was checked by observing the cells periodically under a phase contrast microscope. The spores were pelleted by centrifuging at 2440g for 20 min, washed three times with water, and resuspended in a total volume of 4 mL of buffer (100 mM EDTA/50 mM NaCl, pH 6.9). Lysozyme (Sigma, St. Louis, MI) was added to a final concentration of 1 mg/mL, and the suspension was incubated at 30 °C for 90 min. The spores were washed with water, resuspended in 5% Triton X-100 (Sigma), and sonicated for 1 min in the Bransonic 1210 sonicator (Bransonic, Danbury, CT). The sonicated spores were centrifuged at 772g for 15 min through a 20–60% Percoll (Sigma) gradient. The 60% fraction of the Percoll gradient containing the spores was collected, washed three times with water, resuspended in water, quantitated, and diluted to a final concentration of 10^7 cfu/mL.

Spore Lysis by Sonication. Spore disruption was accomplished by placing 5 mg of 106- μ m glass beads (Sigma) and 75–100 μ L of *B. subtilis* spores in a tube or chamber and then subjecting the tube or chamber to sonication. Three methods to perform sonication were explored. Method 1: A 1.5-mL microcentrifuge tube was held in a Branson 1210 sonicating water bath at 60 W and 67 kHz for 2 min. Method 2: A flat 8 cm \times 8 cm \times 0.1 cm tube (Cepheid, Sunnyvale, CA), consisting of 0.1-mm-thick polystyrene walls, was pressed against a titanium horn of a sonicator probe (Glen Mills, Clifton, NJ) operated at 50 W and 22 kHz for 30 s. Method 3: A spore lysis chamber was interfaced to a minisonicator connected to a Amplified Research model 700A power supply and a Krohne-Hite model 2200 frequency generator. Settings were 40 W and 47 kHz for 30 s.

Spore Lysis by Germination. The procedure was performed as previously described¹⁸ with some modifications. Briefly, spores in water were heat activated for 20 min at 80 °C and then germinated in 10 mM Tris-HCl (pH 8.0), 10 mM L-alanine, and 10 mM CaCl₂ at 37 °C for 40 min. The newly formed vegetative cells were heat labile and were lysed during an initial hold at 95 °C for 15–60 s during PCR analysis.

The Spore Lysis Cartridge and Minisonicator. A spore lysis cartridge, containing a 1.2 cm \times 0.1 cm chamber, was prepared from two acrylic plastic sections. The first section measured 3 cm \times 3 cm \times 0.5 cm with a 1.2-cm centered hole. The second section was 3 cm \times 3 cm \times 1 cm with a centered well (1.2 cm diameter, 0.1 cm depth). Four straight channels (50 mm i.d.) ran perpendicular to the well bottom and through the plastic. The channel ends opposite the well were threaded to accept 0.4-cm plugs. A 1.3 cm \times 0.1 cm \times 0.1 cm annulus surrounded the well and was fitted with an O-ring. The chamber was formed by placing a 3-mm polypropylene membrane over the O-ring and fastening the sections together with four screws. A minisonicator, consisting of stacked piezoelectric disks and a titanium horn, was interfaced with the exposed membrane.

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Real-Time PCR Analysis. Real-time PCR analysis was accomplished using TaqMan probes analyzed on either the ABI Prism 7700 spectrofluorometric thermal cycler (Perkin-Elmer, Foster City, CA) or the advanced nucleic acid analyzer (ANAA).^{10,13} Each reaction (50 μ L for ABI Prism 7700 and 25 μ L for the ANAA) contained buffer (1 \times TaqMan buffer or 1 \times PCR II buffer) (Perkin-Elmer), 0.4 μ M of each PCR primer and probe (NMRC, Bethesda, MD), 5 mM MgCl₂, 0.2 mM each dNTP, 0.05 unit/mL AmpliTaq polymerase (Perkin-Elmer), and sample (10 μ L for the ABI Prism 7700 or 5 μ L for the ANAA). Thermal cycling on the ABI Prism 7700 was performed at 95 °C for 60 s and then 50 cycles of 95 °C for 15 s and 60 °C for 60 s. Thermal cycling on the ANAA was performed at 95 °C for 15 s and then 50 cycles of 95 °C for 4 s and 60 °C for 10 s.

Determining Spore Purity by Flow Cytometry. A 0.5-mL aliquot of sample and 0.5 mL of staining reagent (2.5 μ g of either FITC-labeled goat anti-*B. subtilis* spore polyclonal antibody or FITC-labeled goat anti-*B. subtilis* cell polyclonal antibody, 5×10^3 fluorescent reference beads, 1 \times phosphate-buffered saline (pH 7.4), and 0.05% Tween 20) were mixed in a 2-mL microcentrifuge tube. The tube was incubated at room temperature for 30 min with shaking and analyzed using a BD FACScan flow cytometer (Becton Dickinson Immunocytometry Systems, San Jose, CA). The number of fluorescently labeled bacteria and reference beads were determined from two-dimensional dot plots of light scatter versus FITC fluorescence. The bacteria signal was normalized to the reference beads. Spore purity was expressed as the ratio of spore antibody signal to cell antibody signal.

RESULTS AND DISCUSSION

B. subtilis spores, which serve as surrogates for *B. anthracis* spores, were obtained from the Dugway Proving Grounds. These spores are part of a panel of standards used for evaluating the performance of new instrumentation for bioagent detection. Our initial experiments were designed to determine the effectiveness of using sonication to lyse the spores, since sonication is a widely practiced method to rapidly disrupt a variety of cell types.^{19–23} The mechanism in which sonication forces disrupt cells has been proposed to be gaseous cavitation.^{19–22} In this process, pockets of air form from the dissolved gases in a solution and then rapidly collapse to a portion of the original size, creating high pressure and temperature microenvironments that are damaging to cells. *Bacillus* spores, unlike cells, are very resistant to these damaging forces. Berger and Marr reported that *Bacillus* spores required 1 h of continuous exposure to sonication before significant spore disruption could be measured.²⁴ However, the possibility remained that this disruption time could be significantly reduced by placing glass beads in the suspension. The beads provide more surface area for air pocket formation, resulting in an increase in the number of cavitation events during sonication treatment.^{20–22} Therefore, in our study, spores were sonicated in the presence of glass beads in an attempt to achieve rapid disruption.

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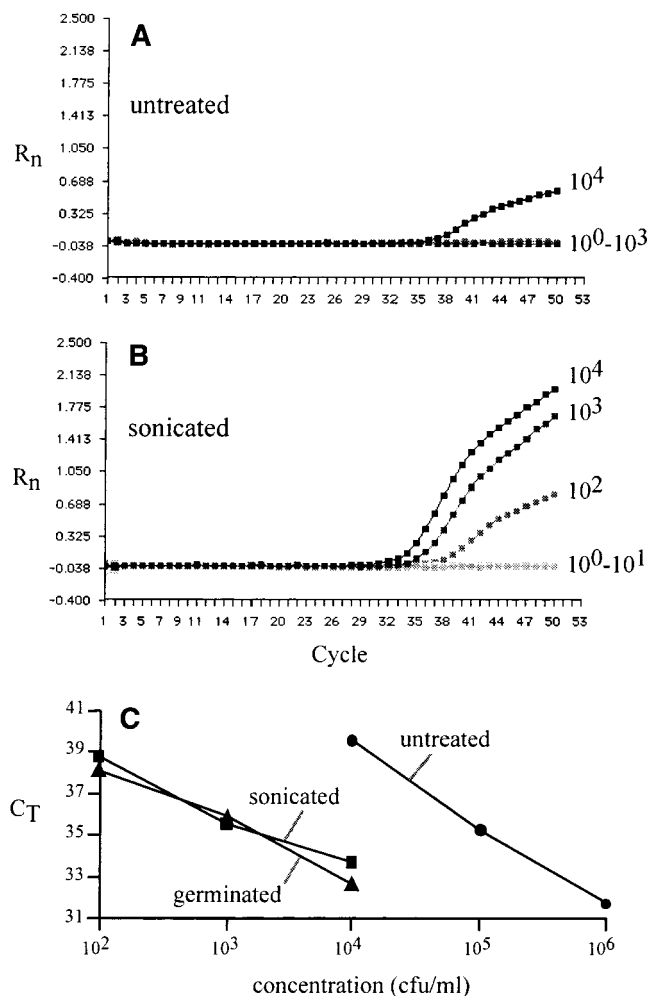


Figure 1. Quantitative real-time PCR detection of *B. subtilis* spores. Tenfold serial dilutions of spores were either left intact (A) or disrupted by sonication (B). Sonication treatment was performed for 2 min using a sonicating water bath. Sample (10 μ L) was subjected to TaqMan analysis on an ABI Prism 7700. The number at the right of each profile indicates spores per milliliter based on colony-forming units in the sample before a 1:5 dilution with PCR reagent. Run time was 130 min. R_n is the normalized signal intensity using Rox dye as a reference. (C) Comparison of sonication and germination treatments for spore disruption. Spores were left intact, germinated for approximately 1 h, or sonicated as described above. Germination induces spores to differentiate into cells, which are susceptible to disruption by heat at the initial 95 $^{\circ}$ C hold step of PCR. TaqMan analysis was performed on an ABI Prism 7700. C_T , threshold cycle at which a significant increase in the positive signal is measured.

B. subtilis spores were serially diluted, placed in microcentrifuge tubes containing glass beads, and incubated in a sonicating water bath for 2–3 min. A bead size of 106 μ m was utilized to permit the beads to agitate in the solution during sonication but settle immediately when sonication was terminated. This avoided the requirement for a centrifugation step. When sonication was completed, samples were taken from the tubes and directly analyzed by quantitative real-time PCR (TaqMan) using the ABI Prism 7700 spectrofluorometric thermal cycler to measure the relative level of available DNA. Real-time PCR detection profiles (Figure 1) obtained from untreated and sonicated spores clearly made evident the dramatic improvement in the PCR detection signals as a result of the sonication treatment. For example, the

treatment reduced the detection limit from 10^4 to 10^2 spores/mL and resulted in the PCR profile of the 10^4 spores/mL sample to display a 4-fold increase in signal amplitude and a 5-cycle reduction in the threshold cycle (C_T) value. The C_T value is the initial cycle number in which the signal significantly increases above the baseline. Comparison of PCR profiles obtained from spores that were either sonicated or germinated, the “gold standard” for spore lysis, confirmed that sonication was effective in lysing the spores (Figure 1C). Samples prepared by either sonication or germination treatments displayed similar C_T values, which were lower than that of intact spores. The acquisition of a positive signal from the intact 10^4 spores/mL sample was not unexpected since DNA generated from bacteria cell lysate produced during the spore preparation process²⁵ becomes associated with the outside of the spores.²⁶ Although this extraneous DNA allows the PCR detection of intact spores, the sensitivity is significantly reduced compared to that of lysed spores.

A 2-min exposure of the spores in the sonicating water bath was the minimal time to achieve greatest enhancement of the PCR signal (data not shown). However, a faster, more efficient approach to disrupt the spores was desired. Therefore, the sonicating water bath was replaced with a sonicator probe. Sonication treatment using a probe typically involves placing the probe directly in the sample. This approach introduces the risk of contaminating the solution with the probe and requires a superfluous volume (>1 mL) of sample. Therefore, sonication of the sample was achieved using a 125- μ L flat tube pressed against the titanium horn of the probe. The tube was well-suited for efficient propagation of the acoustic waves because of its thin polystyrene walls, small volume (100 μ L), and large surface area. Samples of 10^4 spores/mL were placed in the tube, sonicated between 0 and 60 s, and analyzed by PCR. The respective signal profiles and C_T values demonstrated that 30 s of sonication was sufficient to generate the maximum detection signal (Figure 2). For this set of experiments, sonication reduced the C_T value by 10 cycles. Interestingly, this difference in C_T value was greater than that observed in Figure 1 as a consequence of the slow degradation of the extraneous DNA in the stock suspension of spores stored at 4 $^{\circ}$ C.

To understand the role of the extraneous DNA on the PCR signal, the spores were extensively washed with water and high-salt solutions in an attempt to remove this DNA. However, the wash steps failed to weaken the PCR signals of intact spores (data not shown), indicating that this DNA was tightly associated with the spores. It seemed plausible that once the spores were dried down in the presence of the extraneous DNA during the spore preparation, the DNA became irreversibly bound to the spore coat. Therefore, to reduce the level of the extraneous DNA associated with the spores, a progeny of new spores was prepared from the Dugway spores and subjected to a rigorous purification process. The purified spores were quantitated by colony counting and assessed for purity by immunoassays using antibodies specific for *Bacillus* spore and cell proteins. The immunoassays (Figure 3) indicated that bacteria cell lysate proteins were reduced 20-fold in the lot of purified spores, suggesting that these spores, when left intact, would be more challenging to detect by PCR. This was indeed the finding, since the PCR detection limit was

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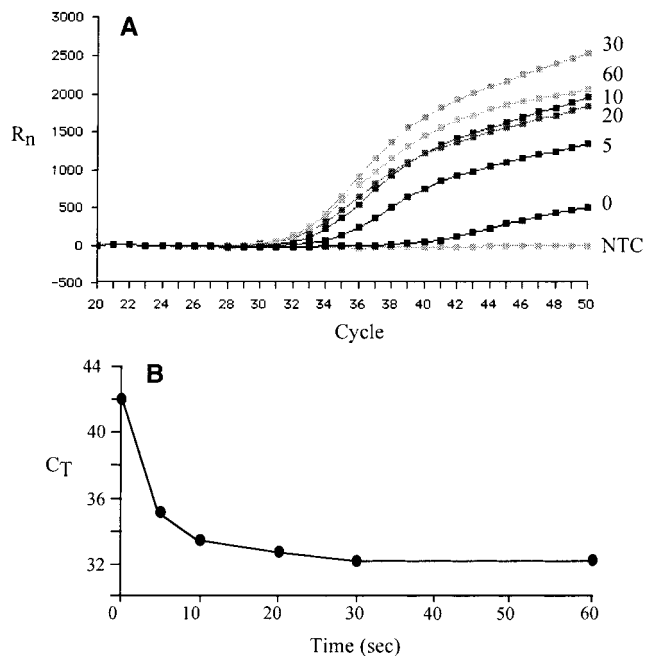


Figure 2. Determining the minimum time for effective spore disruption by sonication. A 125- μ L flat tube containing a sample of spores at 10^4 /mL was pressed against the horn of a sonicator probe for 0, 5, 10, 20, 30, and 60 s. TaqMan analysis was performed on an ABI Prism 7700. R_n is the normalized signal intensity without using Rox dye as a reference. NTC, no template control.

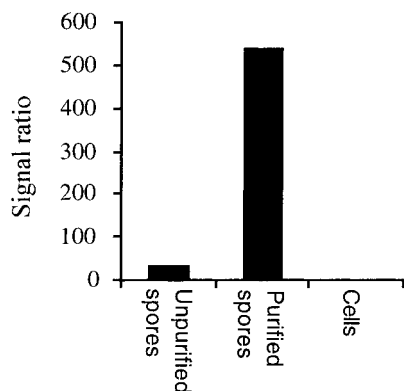


Figure 3. Immunoassays to determine the relative purity of the spore preparations. *B. subtilis* unpurified (Dugway) spores, purified spores, and cells were reacted to polyclonal antibodies specific for either spores or cells. Assays were quantitated using flow cytometry, with the values representing the average of 10 000 events. The signal ratio is the relative amount anti-spore and anti-cell antibodies that were bound to the bacteria. The signal ratio for the cells was less than 1.

elevated to 10^5 (Figure 4A) from 10^4 spores/mL (Figure 1A). However, subjecting the purified spores to the sonication treatment restored the PCR signals to levels similar to those observed for the disrupted, unpurified spores (compare Figures 1B and 4B). Thus, the application of sonication to purified spores improved the limit of detection by three log units, demonstrating that this treatment could be an essential step to rapidly detect a low concentration of highly purified spores in a sample.

First responders and other emergency service providers want small, portable devices that can be brought to the site of a potential biohazard. Therefore, a major goal of this study was to build a prototype spore lysis cartridge with a minisonicator (Figure 5)

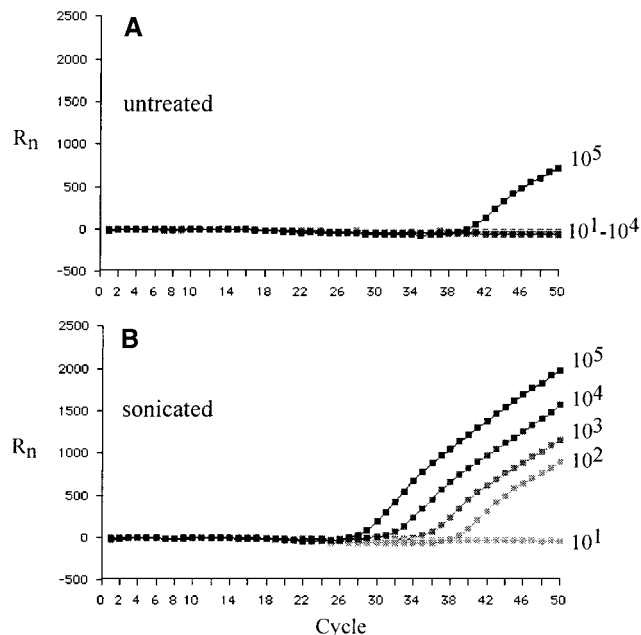


Figure 4. Real-time PCR signal obtained from samples of purified spores. Ten-fold serial dilutions of purified spores were either left intact (A) or disrupted by sonication (B) as described in the legend to Figure 1. R_n is the normalized signal intensity without using Rox dye as a reference.

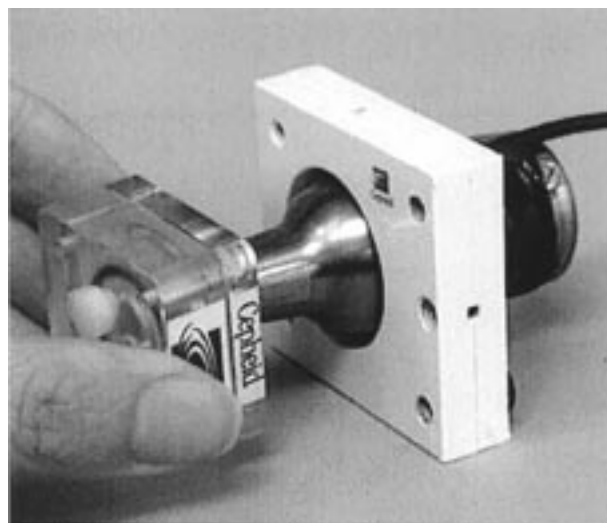


Figure 5. The minisonicator and plastic spore lysis cartridge. Samples of spores were loaded into the chamber through a channel in the cartridge wall and subjected to sonication for 30 s.

that could eventually be integrated into a total analysis system. The large commercial sonicator probe was replaced with a minisonicator that was only a third the size. The minisonicator consisted of a stack of piezoelectric disks that transmitted energy through a 6-cm titanium horn. A 3 cm \times 3 cm \times 1.5 cm acrylic plastic cartridge, containing a 1.2 cm \times 0.1 cm spore lysis chamber to accommodate the minisonicator, was fabricated. One wall of the chamber consisted of a thin polypropylene membrane that made contact with the minisonicator horn. The opposing wall was 0.9-mm-thick acrylic plastic, containing channels for introducing the sample into the chamber.

Samples of the purified 10^5 spores/mL were loaded into the chamber and subjected to 30 s of sonication. Despite its small

size, the minisonicator generated a substantial amount of energy into the chamber. This observation was based on the degree of bead agitation and the rapid rise in chamber temperature measured using a thermocouple. The lysed spores were analyzed on a microchip PCR array instrument, called the advanced nucleic acid analyzer. The ANAA is a portable instrument that offers extremely fast and efficient thermal cycling and real-time monitoring of the reaction.^{10,13} Figure 6 presents data demonstrating that both spore lysis and PCR detection can be completed in 15 min. Spore disruption improved PCR analysis by reducing the detection time by 44% and increasing the signal amplitude by 100% compared to the signal acquired from the sample that was not sonicated.

The spore lysis cartridge and minisonicator are potential elements of a complete sample preparation and nucleic acid analysis system. This system is expected to be man-portable and battery-powered to allow immediate deployment and operation in any location. By utilizing protocols and miniature components that perform with a high rate of speed and efficiency, the size of the system can be minimized, and the earliest characterization of a suspect sample can be achieved.

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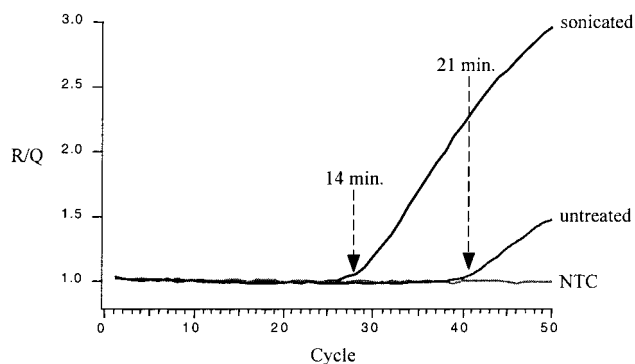


Figure 6. Rapid analysis using the minisonicator and real-time microchip PCR instrument. A sample of purified spores at $10^5/\text{mL}$ was either untreated or disrupted with the minisonicator and cartridge shown in Figure 5. A $5\ \mu\text{L}$ aliquot of sample was added to $20\ \mu\text{L}$ of PCR reagent, and the mixture was analyzed on the ANAA. PCR was performed at a rate of 30 s/cycle. R/Q represents the relative ratio of fluorescence emissions at 530 and 590 nm. NTC, no template control.

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