DISRUPTION OF BACTERIAL SPORES BY SUPERHEATING - A METHOD FOR FAST DNA RELEASE

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ABSTRACT

Bacterial spores are forms of bacteria that are capable of surviving in harsh conditions. We have investigated thermal lysis of the spores and subsequently release of their DNA. We were able to heat water-based samples at ambient pressure to temperatures far above 100 °C without boiling, known as superheating process. It resulted in the breaking of spores in a matter of seconds. Successful release of DNA from spores of the genus *Bacillus* was confirmed by real-time PCR (polymerase chain reaction). This process can be used for release DNA from Anthrax spores, allowing its presence to be detected.

KEYWORDS

thermal lysis, DNA extraction, real-time PCR, superheating, bacterial spores

INTRODUCTION

Polymerase chain reaction (PCR) is the state-of-the-art technique for identifying the presence of bacterial species via their corresponding DNA. Bacterial spores are evolved to withstand harsh conditions, and so breaking their shell to release and subsequently detect their DNA is a difficult task. Conventional methods to detect and identify microorganisms from spore samples are based on spore germination followed by cell lysis, which typically requires a few days.

We have developed a microfluidic system utilizing the miniaturized virtual reaction chamber (VRC) [1]. Here, a droplet of aqueous sample was placed on a microscope coverslip and covered with mineral oil. This VRC was positioned above the micromachined heater with an integrated temperature sensor (Fig. 1). The VRC was separated from the heater by a coverslip.

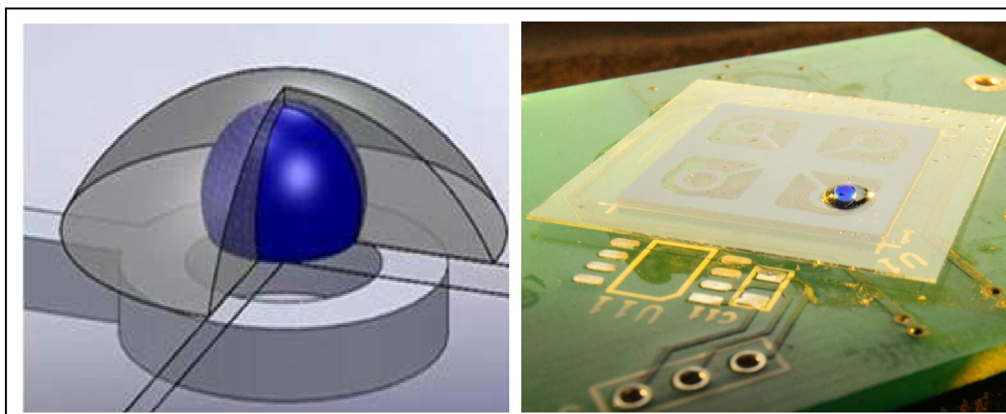

Figure 1: CAD drawing (left) and photograph (right) of a virtual reaction chamber (VRC). The aqueous droplet (blue) containing the sample is encapsulated in oil. The ring-shaped heater/sensor is positioned underneath the sample.

THEORY

Superheating is a well-known phenomenon, requiring three factors: absence of nucleation centers, absence of temperature gradient and no mechanical disturbances, such as shaking [2]. We have discovered that VRC system is well suited for superheating experiments [3]. The VRC does not contain nucleation centers as the coverslip features a scratch-free optical quality surface, and the oil-water interface naturally also does not contain nucleation centers. The temperature gradient is minimal due to the tiny sample volume of 0.2 µL.

The release of DNA from spores can be accomplished by several methods such as germination and cell lysis, spore disruption by laser pulses, or the generation of shear forces by employing ultrasound or glass beads. However, in all cases it is difficult to find a balance between the disruption of spores and the recovery of intact DNA. Thermal lysis of spores could be an attractive method but it requires several hours at 100 °C to destroy the spores. DNA can also be damaged during that process. Currently the most reliable process is also the slowest: germination followed by cell lysis.

Here we have used the superheating system in order to release and subsequently detect DNA from bacterial spores by real-time PCR. Spores of *Bacillus (B.) subtilis* were employed, as a non-pathogenic substitute of *B. anthracis* (Anthrax).

EXPERIMENTAL

A culture of *B. subtilis* cells was grown in liquid broth (LB) medium on a shaker at 150 rpm and 37 °C for 2 days. The culture was then plated on LB agar plates at 37 °C for 5 days for cells to sporulate (Fig. 2a). Spores were transferred from the plates and treated with 10 % hydrochloric acid (HCl) [4]. This treatment destroyed all remaining vegetative cells as well as free DNA to ensure that the *B. subtilis*'s DNA was only presented inside the spores. The efficiency of this step was verified by real-time PCR and no DNA template was found.

The sample was subsequently washed six times with sterile water [4] to remove HCl. The aqueous spore sample with volume between 0.2 and 0.5 µl, was then placed on a hydrophobic microscope coverslip. The sample droplet was then covered with mineral oil. Different droplets were heated up to temperatures ranging between 120 °C and 180 °C, each for 20 s. Images of the samples before and after superheating were captured with an Axiotron II microscope (Zeiss), see Fig. 2b and Fig. 2c.

The sample was then pipetted out from the oil droplet, mixed with 20 µl sterile water and centrifuged. The supernatant was transferred into a new tube and concentrated in a SpeedVac 5301 vacuum centrifuge (Eppendorf), until the final volume of 1 µl was reached. Finally the sample was mixed with a SYBR Green I PCR Master Mix containing TAQ polymerase (Fermentas), forward [TTGGAGAAGTTATCGGGAAAT] and reverse [TAGTTGAAATCCTGAGCCATT] primers, to a final concentration of 50 pM. PCR with this primer set should result in amplicons with a length of 85 base pairs (bp). Real-time PCR was performed using a LightCycler 1.5 (Roche). Additionally, the PCR product specificity was verified by melting curve analysis as well as by gel electrophoresis.

RESULTS AND DISCUSSION

We have proven that the superheating method can release DNA from spore sample in 20 sec and we have found optimum condition for the process.

Purification of spores: An intermediate step in the analysis was to ensure that all DNA released by superheating process originated only from the spores. This was important as the real-time PCR of untreated spore solution had shown that this solution does contain either free DNA, vegetative cells or both. The spore solution was therefore treated with 10 % HCl to destroy all remaining cells together with free DNA molecules. After this procedure no specific amplification by real-time PCR was observed. This confirmed that DNA detected after superheating could only originate from spores. Also, inspection by optical microscope using spore-cell contrast staining methods proved that cells were efficiently removed (Fig. 2a and b).

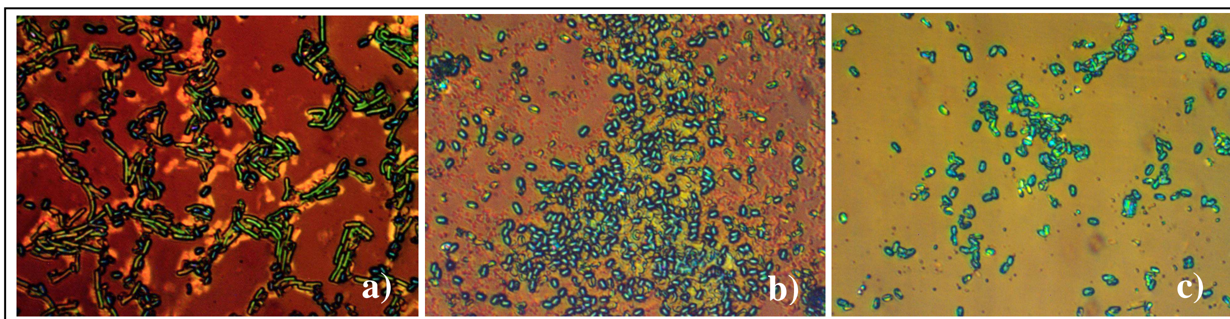

Figure 2: Confocal microscope images (Zeiss Axiotron II) employing a 150x lens. (a) B. subtilis culture after induction of sporulation. Vegetative cells (green) are rod-shaped, spores (blue) are dome-shaped and smaller. (b) 5 days sporulating culture after HCl treatment does not show any vegetative cells. (c) Spores after exposure to superheating at 140 °C for 20 s. The picture indicates that number of intact spores was significantly reduced.

DNA extraction from bacterial spores and PCR: The spore solution was exposed to a range of temperatures from 120 °C to 180 °C. Two experiments were performed for each set of conditions. Comparisons of the images before and after superheating suggests that the spores were indeed disrupted by superheating (Fig. 2b and c). 1 µL solution of each sample was added to PCR master mix and real-time PCR was performed (Fig. 3a). The specificity of PCR was verified by melting curve analysis as well as gel electrophoresis. It was found that the PCR product melting temperature was 82.2 °C ± 0.1 °C, as per analysis by Roche software, and was identical to positive control results. Also, the results of gel electrophoresis showed the DNA products yielded lengths of 85 bp as expected.

As a control experiment, spores were exposed to 100 °C for more than 2 h, with samples taken every 15 min. No specific PCR product was found in these controls, indicating that no DNA was either released from spores or had managed to survive the conditions [5].

Optimization of DNA extraction from bacterial spores: Results from real-time PCR analysis demonstrated that the exposure of spores to superheating successfully opened the spore shells and released the DNA (Fig. 3a). We assume that DNA might be partially damaged during exposure to high temperature. Indeed higher temperature should break more spores in the same time period but consequently destroy more DNA. Based on the critical threshold from the real-time PCR we have found that the highest yield of harvested DNA occured between 160 °C and 170 °C with an exposure time of 20 s(Fig. 3b).

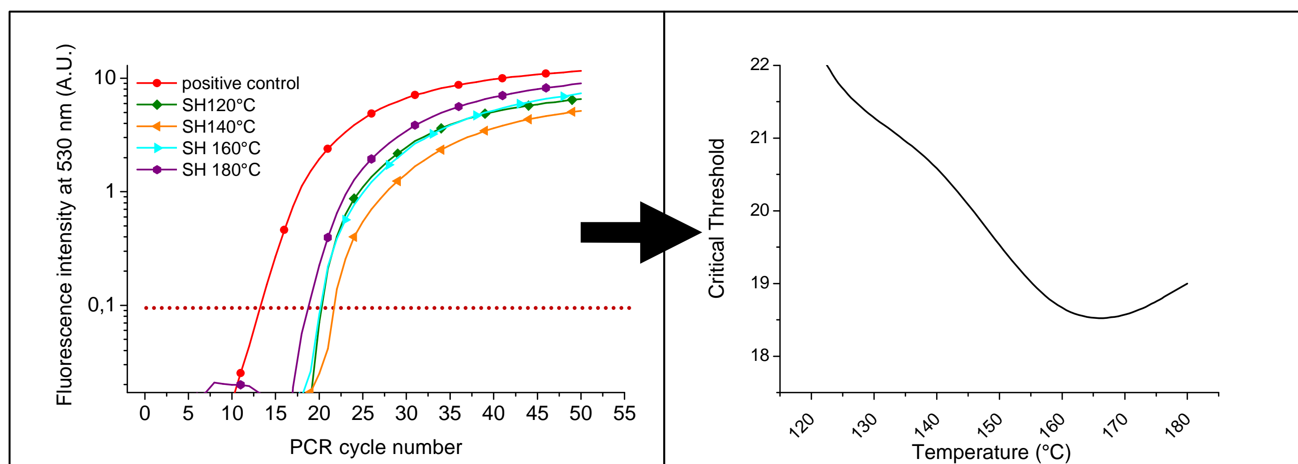

Figure 3a: Real-time PCR data of superheated spore samples. The positive control was cellular genomic DNA. PCR amplification was achieved from all superheated samples with different critical thresholds (dotted red line). The spores after HCl treatment did not show amplification of target DNA.

Figure 3b: Critical threshold (C_T) vs. superheated temperature. The highest yield was found to be at about 165 °C for 20 s exposure time. Below 165 °C the spore breaking process was not as efficient, while at temperatures above 165 °C DNA was damaged to a greater extent.

CONCLUSION

The superheating process has been demonstrated as a simple, fast and effective method for the disruption of spores of the genus *Bacillus* in order to release their DNA for detection. Optimization of DNA extraction has been achieved by varying exposure time and temperature, finding that an exposure time of 20 s at 165 °C temperature results in highest DNA yield. This method can be applied as a front-end tool for sample preparation in order to detect presence of pathogens, such as Anthrax.

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