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Effect of sonic stimulation on Bacillus endospore germination

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One sentence summary: The way Bacillus endospores respond to acoustic sound waves were studied to better understand germination, nosocomial contamination, resistance to stresses and spore communication.

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ABSTRACT

This study investigates the effect of sonic stimulation on Bacillus endospore germination. Germinating endospores in a microtiter plate were exposed to audible sound wave generated by an array of piezoelectric transducers. In situ germination kinetics was measured by terbium-dipicolinate fluorescence assay, optical density measurement and phase contrast microscopy. Fluorescence results revealed that sonic stimulation (5 kHz at 90 dB) promoted the germination speed by 43.7% ± 11.3% and final germination level by 61.7% ± 11.9% of Bacillus atrophaeus. This acoustic energy absorbed by endospores is postulated to change membrane permeability and increase enzyme activities; thereby, expediting the germination process. This also raises the likelihood of dormant endospores undergoing germination because of a rapid release of unidentified chemical mediators for quorum sensing. On the other hand, acoustic effect was not observed in B. subtilis endospores. This may be attributed to the different spore aspect ratio, 1.43 ± 0.05 for B. atrophaeus and 2.02 ± 0.08 for B. subtilis, which results in a difference in specific absorption rates towards audible sound waves. Our results demonstrate the modulation of endospore germination by an external field to shed light on germination mechanism and cell-wave interaction.

Keywords: endospore germination; long-distance cell communication; cell response to physical fields; acoustic stimulation

INTRODUCTION

Endospores are highly dehydrated cells formed under external stress by certain bacterial genera, such as Bacillus and Clostridium, in order to survive a harsh environment (Gould 2006). They are metabolically dormant and very resistant to environmental stress factors such as UV radiation and high temperature. Some bacilli endospores, such as Bacillus cereus, are causative agents of foodborne disease and food spoilage (Granum and Lund 1997; Arnesen, Fagerlund and Granum 2008). Endospores of B. anthracis are a major bioterrorism threat (Ellerbrok et al. 2002; Higgins et al. 2003). While such endospores can remain in their dormant and resistant state for extended periods up to millions of years, they return to a vegetative state rapidly through germination when exposed to favorable environmental conditions. Germination is a crucial step in reviving dormant endospores, during which the endospore’s dormancy, and extreme resistance are concomitantly lost. Germinated endospores are more susceptible to inactivation, which underlines the importance of germination in nosocomial contamination, food poisoning and environmental microbiology. Germination can be triggered by environmental cues such as nutrients, water, and laboratory-controlled non-nutrient-based chemicals such as lysozyme (Setlow 2003). Lots of work has been carried out to investigate the trigger conditions (Setlow, Cowan and Setlow 2003), heterogeneity (Chen, Huang and Li 2006; Lopez, Vlamakis and Kolter 2009; Setlow, Liu and Faeder 2012), mechanisms (Paredes-Sabja, Setlow and Sarker 2003) and non-nutrient-based chemicals. The recent advancement of sonic stimulation to promote germination could provide a new strategy to address nosocomial contamination.
and quorum sensing (Zhang et al. 2011; Webb et al. 2012) of germination through biochemical and electrochemical methods (Setlow and Kornberg 1970; Liu et al. 2007). But there is a paucity of work on probing the interaction of germination with physical factors, such as electric field (Hamilton and Sale 1967; Pol et al. 2001), magnetic field (Moore 1979) and sound wave stimulation (Matsuhashi et al. 1998). Here, we take a novel approach to look at the effect of audible sound on endospore germination.

Nutrient-triggered germination takes place when specific germinants bind with germinant receptors. A cascade of events starts by the release of monovalent cations from the spore core. Pyridine-2,6-dicarboxylic acid (dipicolinic acid, DPA), mostly chelated with divalent Ca$^{2+}$, is released from the spore core and subsequently displaced by water. This released Ca-DPA binds with cortex-lytic enzymes (CLEs) located at the cortex-coat boundary, leading to hydrolysis of the spore cortex, further release of Ca-DPA and water influx. Metabolism and macromolecular synthesis follow rehydration of spore core (Setlow 2003). Hydrostatic pressure, a kind of mechanical force, induced germination by activation of GerA and GerB receptors at 100 MPa (Wuytack et al. 2000) and activation of Ca-DPA channels at 550 MPa (Paidhungat et al. 2002).

Bacillus germination is a highly coordinated process regulated via quorum sensing. Quorum sensing is a cell–cell communication mechanism. By detecting the signaling molecules produced, bacteria are able to count their own numbers and synchronize their behaviors when the signaling molecules concentration passes threshold values (Bassler and Losick 2006). In this context, quorum sensing regulates the sporulation process of B. subtilis. It was reported that two peptides (ComX and CSF) mediated the quorum sensing control of competence and sporulation (Dunny and Leonard 1997). Bacillus physiology is closely tied with cell numbers. Sporulation occurs only poorly at low cell density, even if cells are starved (Miller and Bassler 2001). Caipo et al. (2002) found that B. megaterium endospore germination was influenced by inoculum size. Zhang et al. (2011) reported that communication between endospores during germination exists in which adjacent endospores tend to elicit more synchronized behavior. Audible sound waves have been suggested as an alternative means of communication among bacteria (Nikolaev 2000; Nikolaev, El‘Registan and Desu 2007) and to affect growth rate and biomass production of microorganisms (Nikolaev et al. 2003). For instance, Gu and coworkers reported that audible sound treatment at 1, 5 and 10 kHz, 90 dB, showed an increase in the number of colonies formed by endospores of B. subtilis (Gu et al. 2003) in antibiotic containing media, even when interrupted by barriers, such as 1.2-mm-thick plastic plates and 2-mm-thick iron. This growth promotional signal was transmissible through air over a distance of up to 40 cm. The physical factor involved was suggested to be of a sonic nature, probably generated by vibration of some intracellular organelles such as cytoskeletons, membranes and chromosomes. They further demonstrate that certain frequencies of sound were able to promote the growth of B. carboniphilus in non-permissive stress agar (Matsuhashi et al. 1998). Recently, Gu and coworkers reported that audible sound treatment at 1, 5 and 10 kHz, 90 dB, showed an increase in the number of colonies formed by Escherichia coli under normal growth conditions (Gu et al. 2010). They suggested that sound stimulation induced intracellular fluid motion, affected plasma membrane protein structure, and facilitated efflux of water, which resulted in membrane trafficking modulation and acceleration of metabolic activities. Gu et al. (2013) further found that a sound field with 5 kHz 100 dB increased the activities of super oxide dismutase and catalase. There were also reports on the ultrasonic stimulation effect of bioprocesses (Kwiatkowska et al. 2011). Ultrasound with a specific intensity is able to increase the enzyme activity. Apart from microbes, audible sound wave can also greatly influence the growth of plants. It was reported that sound wave was able to enhance the metabolism (Yi et al. 2003b), change the physical state of plasmalemma lipid and secondary structure of plasmalemma protein (Yi et al. 2003c), accelerate the synthesis of RNA and soluble protein (Wang et al. 2003), and increase the activities of plasmalemma H$^+$-ATPase (Yi et al. 2003a) in chrysanthemum roots. Although bacteria are considerably different from plants, they share many of the same underlying biochemical processes. On the other hand, Pelling et al. (2004) demonstrated that cell walls of living Saccharomyces cerevisiae exhibited local temperature-dependent nanomechanical motion at characteristic frequencies ranging from an audible range between 0.8 and 1.6 kHz. They suggested that this nanomechanical process was metabolically driven from a concerted action of molecular motors, proteins which might be part of a communication pathway or pumping mechanism. But the underlying mechanism is still not well understood.

Driven by previous reports, we would like to explore the effect of acoustic wave on endospore germination. Endospores of B. atrophaeus and B. subtilis were subjected to acoustic wave stimulation of 5 kHz 90 dB using piezoelectric buzzers. Germination kinetics was measured by terbium-dipicolinate (Tb-DPA) fluorescence assay, optical density and phase contrast microscopy.

**MATERIALS AND METHODS**

**Materials and setup**

*Bacillus atrophaeus* (ATCC 9372) and *B. subtilis* (ATCC 6633) endospores were cultured, sporulated, purified and harvested as previously reported (Yung and Ponce 2008). An endospore purity of 99.9% was attained (see Supporting Information). Endospore suspensions were stored at 4°C in the dark. All chemicals were purchased from Sigma Aldrich (St. Louis, MO). Experimental setup is illustrated in Fig. 1. Piezoelectric transducers were purchased from Welfare Ltd. (Hong Kong). Sound pressure level was measured by a sound pressure meter (Center Technology Corp., Taiwan).

**Endospore germination**

The germination experiment was carried out inside an enclosure (Fig. 1) and continuously stimulated by 90 dB sound
waves with a frequency of 5 kHz. Germination was conducted at room temperature and 37°C for *B. subtilis* additionally. Tb-DPA fluorescence assay, optical density, and phase contrast microscopy were carried out. Control experiments were conducted as appropriate.

**Tb-DPA fluorescence assay**

Endospore suspension (final concentration $1.6 \times 10^8$ cells ml$^{-1}$) was centrifuged to remove supernatant DPA, resuspended in Tris-HCl buffer (50 mmol l$^{-1}$, pH 7.6) and transferred to 96-well flat-bottom black polystyrene plates (SPL Life Sciences, South Korea) containing L-alanine (0.25 mol l$^{-1}$), TbCl$_3$ (50 μmol l$^{-1}$) and Tris-HCl buffer. Control experiments included samples without germinant, samples without bacterial endospores and samples without either germinant or endospores. Fluorescence emission spectra ($\lambda_{ex} = 278$ nm, $\lambda_{em} = 532-557$ nm) were recorded using a MicroMax microplate reader in the Fluorolog-3 spectrofluorometer (Horiba Jobin Yvon, Edison, NJ, USA). A 495 nm long-pass filter was placed along the emission path to eliminate second-order diffraction. The control plate followed the same procedure without sound stimulation.

**Optical density measurement**

Endospore suspension was washed and resuspended in Tris-HCl buffer (50 mmol l$^{-1}$, pH 7.6) with an OD$_{600}$ of 1.0 with L-alanine (0.25 mol l$^{-1}$) in 96-well flat-bottom transparent polystyrene plates (SPL Life Sciences, South Korea). The OD$_{600}$ values were measured by an Elx800 absorbance microplate reader (BioTek, VT, USA) every 10 min. Samples were thoroughly mixed by vortexing before measurement.

**Phase contrast microscopy**

Endospores samples were taken every 30 min from 96-well plate during optical density measurement and observed under a Ti-U microscope (Nikon, Japan). Every 30 min, the percentage of phase dark body was counted and calculated based on over 500 individual endospores.

**ATP bioluminescence assay**

Endospores of *B. atrophaeus* ($1 \times 10^8$ cells ml$^{-1}$) were germinated with L-alanine (25 mmol l$^{-1}$) in Tris-HCl buffer (pH = 7.8 at 25°C) at room temperature with or without sound stimulation (5 kHz, 90 dB). ATP was extracted and measured by the firefly-luciferase assay (Sigma, MO, USA) using a luminometer (SystemSure Plus, Hygiena, CA, USA) from endospore samples at various time points of germination process as reported (Setlow and Kronberg 1970).

**Kinetic modeling and statistical analysis**

The germination time course data were fit by following model (Peleg and Normand 2013):

$$P(t) = P_{\text{asym}} \left[ 1 - \exp \left( -t/t_c \right)^m \right]$$

$P_{\text{asym}}$, $t_c$, and $m$ stand for final germination level, time for reaching 63.2% of the asymptotic value, and shape factor of the germination curve, respectively. We further define $k$, the maximum slope, as the germination speed in the analysis part,

$$k = \frac{P_{\text{asym}} \ln (1 - \frac{1}{m})}{t_c \exp \left( \frac{1}{m} - 1 \right)}$$

Unpaired Student’s t-test was conducted. A level of $P < 0.05$ was accepted as statistically significant.

**RESULTS**

**Germination of *B. atrophaeus* endospore**

Figure 2A shows the germination time course of *B. atrophaeus* endospores subjected to sound stimulation (5 kHz, 90 dB). In the first 10 min, the sound-stimulated samples exhibited a 43.7% ± 11.3% faster germination rate as measured by DPA release. The fluorescence signal leveled off after 50 min. The Tb-DPA fluorescence intensity of samples is 61.7% ± 11.9% higher than the control after 2 h of germination. The curve fit results show statistically significant ($P < 0.05$) differences on both final germination level and germination speed.

Figure 2B shows the optical density measurement. In the first 10 min, the sample exhibited 39.2% ± 16.8% faster germination rate as measured by OD$_{600}$. The fluorescence intensity kept increasing, with a comparable rate as in the control. The optical density loss of samples was 10.0% ± 4.7% higher than the control after 2 h of germination. The curve fit results show a statistically significant ($P < 0.05$) difference on both final germination level and germination speed. Results of phase contrast microscopy and optical density measurement were in good agreement (Fig. S2, Supporting Information).

The luciferin-luciferase bioluminescence assay measured a higher ATP level in the sound-stimulated sample (Fig. 3). In the first 40 min, the ATP production rate of sample was 2.4 times higher than the control. The sample ATP amount plateaued at 40 min and decreased by 23% afterwards while control ATP production kept increasing after 40 min.

**Germination of *B. subtilis* endospore**

Germination time course of *B. subtilis* subjected to 5 kHz sound stimulation were detected by Tb-DPA fluorescence assay and optical density at 600 nm. The whole germination process showed no statistically significant differences (P > 0.05) between samples and controls under both room temperature and 37°C. The germination kinetics at 37°C is shown in Fig. 2C, D to highlight the contrast between these two species. The final germination level ($P_{\text{TbDPA}} = 0.42$, $P_{\text{OD}} = 0.48$) and germination speed ($P_{\text{TbDPA}} = 0.37$, $P_{\text{OD}} = 0.47$) are determined and compared through curve fitting and t-test. The result of phase contrast microscopy was consistent with optical density measurement.

**DISCUSSION**

Our results show that sonic wave at 5 kHz promoted the germination of *B. atrophaeus* endospore in terms of final germination level and germination speed (Fig. 4A). Three major processes were tracked during endospore germination: DPA release, water influx and ATP production. Amount of DPA released and its releasing kinetics are widely used to study endospore germination. The precise mechanism of DPA release is not clear yet. But evidence shows that the Ca-DPA efflux is gated by channels formed with SpoVA proteins in the inner membrane of endospore (Setlow 2013). Cortex peptidoglycan structure also
Figure 2. Germination time course of \( B. \) atrophaeus (A) and (B) and \( B. \) subtilis (C) and (D) endospore with L-alanine. Endospores were germinated with and without 90 dB 5 kHz acoustic wave stimulation. Germination was detected by Tb-DPA fluorescence assay (A) and (C) and optical density at 600 nm (B) and (D) for two hours in 96-well plates. Time course data were fitted to the model outlined in Materials and Methods. The final germination level \( P_{\text{asym}} \) and germination speed \( k \) were evaluated and compared. Error bars indicate the standard deviation (\( n \geq 6 \)). Asterisk indicates the observed statistical significance (\( *P < 0.05 \)).

Figure 3. ATP production during germination of \( B. \) atrophaeus endospore. Endospores were germinated in L-alanine with or without 90 dB 5 kHz sonic wave stimulation.

ATP level was also measured during germination. Dormant endospores have no detectable metabolic activity and contain almost no stored ATP (Setlow and Kornberg 1970). In the early stage of germination, ATP is derived mostly from stored AMP and ADP using endogenous energy. Setlow et al. (2009) suggests that ATP generation follows endospore cortex hydrolysis when there is sufficient uptake of water into spore core. Results here show that endospores uptake water faster in the first 30 min. This acceleration of water influx is not clear and may be caused by (i) an elevated level of Ca-DPA binding with CLEs or (ii) sonic wave stimulates the enzymatic activity of CLEs leading to faster cortex hydrolysis (Kwiatkowska et al. 2011).
that acoustic wave stimulation increased the rate and amount of ATP generation. Presumably, the acoustic waves accelerate a series of early stage germination activities, which in turn accelerates the water intake and ATP generation. In addition, ATP generation is an enzymatic process, which may be expedited by acoustic stimulation (Syroeshkin, Bakeeva and Cherepanov 1998).

In Fig. 4A, sonic stimulation was shown to cause higher fluorescence intensity and a more significant drop in optical density, indicating more endospores undergoing germination. It may be due to the quorum sensing in a population. Report of Zhang et al. (2011) suggested that adjacent endospores tend to elicit more synchronized germination. But the molecules caused this effect are still unknown. It is possible that acoustic wave accelerates the release of those chemical mediators from endospores. A high local concentration of signaling mediators triggers off more germination events. It is also possible that endospores are actually communicating through sonic wave in a similar way as Matsuhashi et al. (1998) observed. The acoustic signal applied to spores was acting as communication signal to stimulate spore germination. The underlying principle of acoustic communication remains unclear.

No promotional effect was observed in B. subtilis endospore germination (Fig. 4A) under 90 dB 5 Hz stimulation. Under phase contrast microscopy, the dimensions of B. subtilis endospores are clearly different from B. atrophaeus. Carrera et al. (2007) reported that the mean aspect ratio (length/diameter) of B. subtilis (2.23 ± 0.24) is the largest among other bacilli species (B. atrophaeus: 1.85 ± 0.19). Absorption coefficient is largely governed by the shape of micrometer-sized particles. Over a frequency range of 150–1500 kHz, absorption coefficients for disk-like and needle-shaped particles were reported to be less than their spherical counterparts by 5%–27% and 24%–41%, respectively (Blue and McLeroy 1968). From Fig. 4C, B. subtilis endospores are mostly needle-shaped (aspect ratio 2.02 ± 0.08) with lower absorption coefficients compared with round-shaped B. atrophaeus endospores (aspect ratio 1.43 ± 0.05) (Fig. 4B). But when we doubled the sound power intensity (93 dB), there is still no promotional effect (P > 0.05). The intensity was not further increased because it may have heating effect and induce evaporation or atomization. Meanwhile, it is also possible that B. subtilis did not respond to this frequency because of its morphology differences. The resonance frequency of a spherical shell, if we regarded it as a spherical cap, is related to the radius of the circular edge, height of the dome and the shell thickness (Fletcher 1992). The frequency screening experiments will be conducted in a high-throughput microfluidic device in the future.

This study demonstrates that sonic stimulation in the form of a mechanical wave expedites the germination level and rate of round-shaped B. atrophaeus endospores but not needle-shaped B. subtilis endospores. Other parameters such as frequency dependency study and germinant dependency study are yet to be investigated. Single endospore measurement with in situ real-time detection will be made possible by developing a microfluidic platform with ultrasensitive MEMS sensors.

**SUPPLEMENTARY DATA**

Supplementary data are available at FEMSLE online.
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Conflict of interest. None declared.

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