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Single-cell analysis reveals microbial spore responses to microwave radiation

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To determine the effects of microwave radiation at the molecular level as well as on the germination, growth and morphology of dry spores at the single-cell level. Dry *Bacillus aryabhattai* MCCC 1K02966 spores were microwave-treated at different powers and characterized using single-cell optical technology. As determined by laser tweezers Raman spectroscopy, the Ca^{2+}-dipicolinic acid content increased and nucleic acid denaturation occurred in response to microwave treatment. Live-cell microscopy revealed that the germination and growth rates decreased as the microwave power increased. With respect to morphology, atomic force microscopy (AFM) demonstrated that spores became wrinkled and rough after microwave treatment. Furthermore, spores became smaller as the microwave power increased. Microwave treatment can damage DNA, and high-power microwaves can inhibit the germination of spores and reduce spore volumes. These results provide a new perspective on the responses of living single cells to microwave radiation and demonstrate the application of various new techniques for analyses of microorganisms at the single-cell level.

Keywords: Single-cell analysis; Bacillus; spore; live-cell microscopy; laser tweezers Raman spectroscopy.

1. Introduction

Rapid advances in microwave technology for information transmission, military applications and radar detection have profoundly affected living systems.[1–4] The effects of microwave radiation and downstream consequences have been of great interest in biological and biomedical research.[5–7] Microwaves, with a frequency range of 300 MHz to

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300 GHz, are nonionizing with high absorption and penetrability.[2,8,9] Whether this kind of radiation directly breaks chemical bonds in molecules like DNA depends on the energy, which is also important for heating and penetrating microorganisms.[10–12] Among these features, the absorption and penetrability of microwaves are remarkable, with the ability to penetrate cells and thereby affect biological activities.[13–15] Therefore, environments, where microwaves are distributed, have great impacts on organisms and it is important to understand the responses of microorganisms like spores to these conditions. Spores are the dormant state of bacteria under nutrient deficiencies; dormancy functions to protect against extremely harsh environments, such as microwave radiation and toxic chemicals, *via* special multi-layer structures and low water content.[16–18] These protective structures could lock DNA stably and protect against toxic substances, involving minimal metabolic activity.[16,19,20]

Many studies have evaluated the effects of microwaves on spores, including the following properties: (i) Spore viability, (ii) the release of molecules and (iii) spore structures captured by electron microscopy. These studies have demonstrated that microwave radiation can reduce the viability of spores at a sufficient power and temperature. *Bacillus licheniformis* spores were killed after 2 min when exposed to microwave radiation with a power of 2000 W.[21] *B. cereus* spores and *Clostridium difficile* spores were inactivated by microwave irradiation at 800 W for 1 min.[21,22] *Paenibacillus larvae* spores were killed by microwave irradiation at 850 W for 2 min.[23] Furthermore, the significant leakage of proteins and DNA from spores due to structural damage has been observed under 2.0 kW microwave and transmission electron microscopy has revealed that microwave irradiation affects spore cortex hydrolysis and swelling and ruptures the coat and inner membrane.[24] The cortex of irradiated spores was not affected and DPA release from irradiated spores was undetectable under 100 W.[25] All of these previous studies have focused on spore suspensions. The effects of microwave radiation on dry spores are still unclear. In addition, the methods used in most previous studies, such as transmission electron microscopy and scanning electron microscopy, could damage biological samples.[26] However, the response of bacterial spores to microwave radiation is heterogenous, group behaviors can't reveal individual heterogeneity.[27] Differences at the single-cell level are ignored and effects on the growth and surface morphology of single cells have not been determined.

In this study, we evaluated the responses of dry spores to microwave radiation by non-destructive single-cell optical technologies. We examined the changes in spore molecules after microwave treatments using laser tweezers Raman spectroscopy (LTRS), characterized the germination and growth of treated and untreated single spores by live-cell microscopy and characterized the morphology of spores by atomic force microscopy (AFM). The results of this study improve our understanding of the responses of dry spores to microwave environments, including molecular and morphological changes and provide new insight into the effects of microwave radiation at the single-cell level.

2. Materials and Methods

2.1. *Strains, media, and spore preparation*

Bacillus aryabhattai MCCC 1K02966 spores were used, which are highly resistant to radiation due to β-Carotene and relatively dormant resulting in a low germination; these spores were collected from sampling areas in the Yellow Sea near China (77° 31′N and 69° 190′ W in July 2010) from a depth of about 2700 m. Spores of strain 2966 were prepared on 30% oligotrophic 2216E medium agar plates at 37°C. After incubation for five days, spores were purified as described previously.[28] All purified spores were stored at 4°C in water protected from light and were $> 98\%$ free from growing and sporulating cells, germinated spores and cell debris, as observed by phase-contrast microscopy.

2.2. *Spore microwave treatment and measurements of internal molecules*

The Discover SP Microwave Synthesizer (CEM Discover SP; CEM, Matthews, NC, USA) was used, providing a relatively stable electromagnetic field and adjustable microwave power, temperature and pressure within a certain range. Spores (30 μl of $\sim 10^8$ spores ml^{-1} in water) were dropped on small quartz tablets and evenly distributed and then the

quartz tablets containing spore suspensions were placed in a vacuum machine for 15 min of vacuum pumping to dry spores. The quartz tablet containing the dry #2966 spores was placed in a tube (volume: 35 ml) and the tube was placed in the microwave system for 1 min at different powers (50 W, 100 W, and 150 W), the spores untreated served as control. All treatments were carried out at the same temperature and pressure (25°C and 15 PSI). After the microwave treatments, the spores were suspended in distilled water to a concentration of $\sim 10^7$ spores ml^{-1} and stored at 4°C for subsequent analyses of viability, live-cell imaging, and being applied to be examined by LTRS.

Molecular changes in #2966 spores were evaluated by Raman spectroscopy using LTRS. The LTRS system used in this study adopts a 532 nm laser beam to randomly trap single cells suspended in water and excites to produce Raman scattering acquired by a charge-coupled device (CCD). Only 2.4 mW laser power was used to avoid spore damages and the integration time was 30 s. The Ca^{2+}-dipicolinic acid (CaDPA) contents of spores were determined from the intensity of the CaDPA-specific Raman band at 662, 825, and 1017 cm^{-1} relative to that of CaDPA standards.[29,30] The nucleic acid contents of spores were determined from the intensity of the nucleic acid-specific Raman band at 782 cm^{-1} relative to that of nucleic acid standards.[31]

2.3. *Live-cell imaging of spore viability and germination as well as the outgrowth and growth of single spores incubated in a 100% eutrophic 2216E medium agar pad*

For analyses of the germination, outgrowth and growth of untreated or microwave-treated spores, live-cell microscopy (N-storm; Nikon, Toyko, Japan) was used to lock-in live-cell images in focus. A small drop (1 μl of $\sim 10^7$ spores ml^{-1} in water) of #2966 spores was spread on the surface of a microscope slide, dried in a vacuum desiccator for 10 min and mounted on a microscope sample holder kept at 25°C. Approximately 250 μl of melted 2216E agar at ~60°C was added to the top of the spores on the sample cell to form an agar pad with a thickness of ~3 mm. Some agar on the side of the pad was removed to form a small hole to contain air and then a coverslip was applied to seal the top of the agar pad, thus preparing the spores for bright-field microscopy. A digital CCD camera (12 bits, 2044×2048 pixels) was used to record bright-field images of the spores on the sample cell at a rate of 1 frame every 60 s for 4 h. In these measurements, ~250 single spores were monitored for each microwave condition.

The viability of control and microwave-treated dry spores was also measured by comparing images obtained before and after incubation using a microscope, enabling clear observations of the survival of spores and reducing errors. The specific operation details are described above. In viability measurements, ~900 single spores were recorded for each microwave condition.

2.4. *AFM imaging of spores*

Droplets of the #2966 suspension (0.5 μl of $\sim 10^7$ spores ml^{-1} in water) were deposited directly onto poly-coated quartz tablets and allowed to settle for 30 min, after which the samples were dried in air. The tablets were then added to the tube for microwave treatment at 50 W, 100 W, or 150 W for 1 min, using the Discover SP Microwave Synthesizer, which was set to 25°C and 15 PSI. After microwave treatment, each sample was mounted onto a sample holder for imaging in air.

Images were collected by AFM using the NanoWizard 4 BioScience atomic force microscope (Bruker, France) operated in AC mode. AFM probes consisting of silicon tips on silicon nitride cantilevers were used for imaging, which was performed at a resonant frequency of ~320 kHz and elastic constant of 42 $N\,m^{-1}$. All images were scanned with 512 pixels per line at a scan rate of 0.5–1 Hz. All imaging operations were conducted at room temperature.

3. Results

3.1. *Spore survival and CaDPA content after microwave treatment*

After exposure to microwave radiation at 50 W, 100 W, and 150 W, the survival rates of #2966 spores decreased compared to untreated spores (Fig. 1(a)). Although the microwave treatments

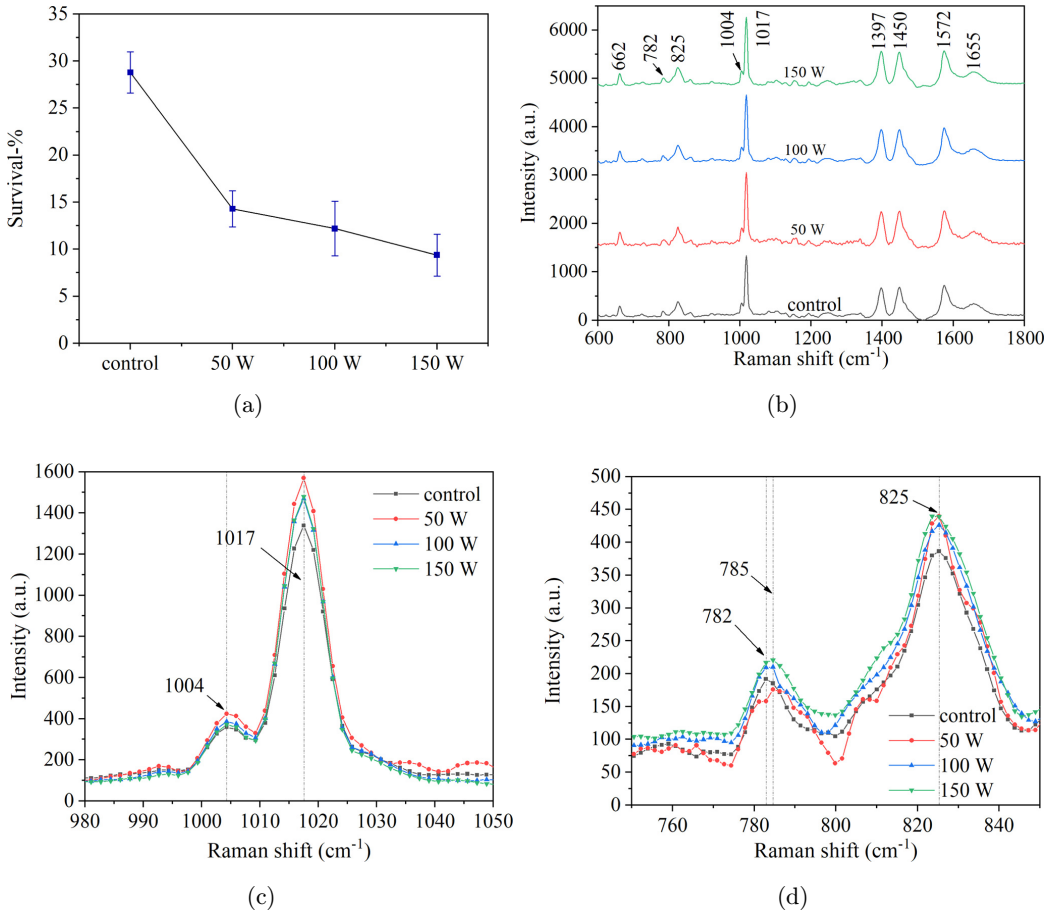


Fig. 1. Effects of microwave treatments on the dry #2966 spores. (a) Percentages of spores that survived as a function of microwave power at 25°C were determined as described in Methods. (> 900 single spores were counted in each treatment). (b) Raman spectra of single #2966 spores after various power microwave treatments, that were the average spectra from 30 single spores. (c) Raman spectra of single #2966 spores in lipid and CaDPA band regions. (d) Raman spectra of single #2966 spores in nucleic acid band regions. a. u., arbitrary units.

were carried out for a short duration (1 min), the difference in spore survival between microwave-treated and untreated spores was significant.

To evaluate the effects of microwave treatments on spores at the molecular level, the average Raman spectra from 30 single spores were obtained by LTRS for a comparison of #2966 spores that were untreated or treated at 50 W, 100 W, and 150 W (Fig. 1(b)). Slight Raman spectral changes in #2966 spores suspended in water were detected after microwave treatments. Under microwave treatment, bands at 662, 825, and 1017 cm^{-1} due to CaDPA and at 1004 cm^{-1} due to lipids increased slightly (Fig. 1(c)) and similar results were obtained at 662, 1397, and 1572 cm^{-1}, also due to CaDPA, showing that small changes in the spore core around CaDPA. In particular, the Raman peak at 782 cm^{-1} due to nucleic acids shifted at 50 W, 100 W, and 150 W compared to that in the control, indicating that microwave can damage DNA. Multiple studies with *B. subtilis* spores have used Raman spectroscopy to validate DNA damage by other exposures e.g. γ-ray.[32] We also observed obvious differences in single-cell Raman spectra compared to untreated samples, 12, 14 and 18 single spore Raman spectra showed a peak shift at 782 cm^{-1} under microwave treatment at 50 W, 100 W and 150 W, respectively, among 30 microwave-treated spores (Fig. 1(d)), indicating that nucleic acid denaturation occurred and became worse as the microwave power increased. In addition, as the microwave power increased, the standard deviations of the I_{662}/I_{1017} ratio decreased and the I_{825}/I_{1017} ratio increased (Table 1), revealing that a higher power changed the spore core around CaDPA.[33]

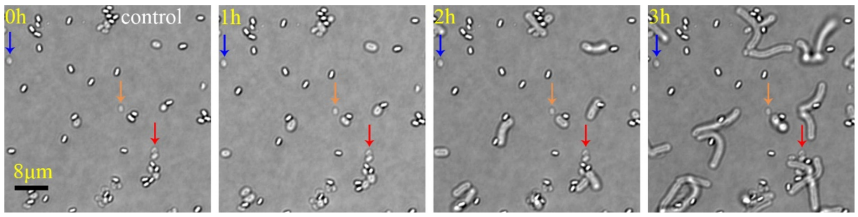
Table 1. Ratios of intensities of the Raman peaks at 662, 825, and 1017 cm^{-1} in multiple individual untreated and microwave-treated #2966 spores.[a]

	Intensity ratio (AU[b]) for treatments parameters:			
Raman peaks	Control	50 W	100 W	150 W
I_{662}/I_{1017}	0.151 ± 0.012	0.156 ± 0.011	0.145 ± 0.010	0.148 ± 0.009
I_{825}/I_{1017}	0.224 ± 0.018	0.225 ± 0.013	0.232 ± 0.015	0.240 ± 0.013

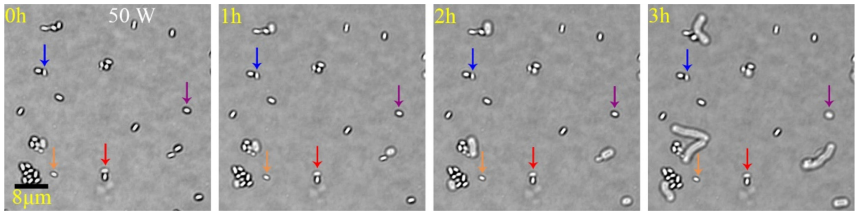
Notes: [a]I_{662}, I_{825}, and I_{1017} values were determined by LTRS for 30 individual untreated and microwave-treated spores and averaged. Standard deviations are indicated. [b]AU, arbitrary units.

3.2. *Comparison of germination, outgrowth and growth in untreated and microwave-treated spores*

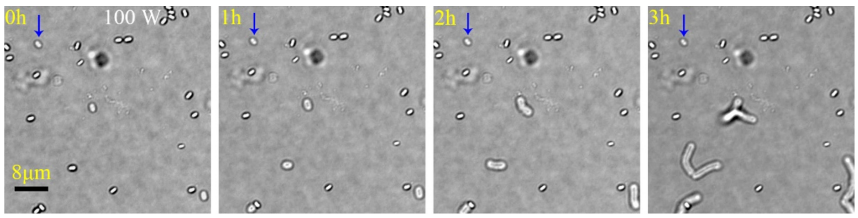
To analyze the effects of microwave treatments on spore germination, outgrowth and growth, #2966 spores with and without microwave treatments were cultured on a 100% eutrophic 2216E medium agar pad on a microscope sample cell at 25°C and bright-field images were recorded every 60 s for 4 h (Figs. 2(a)–2(d)), showing no significant difference in treated spores at different microwave powers. In both microwave-treated and untreated groups, there were spores that released CaDPA at the beginning but did not continue to grow or germinated very late. However, as the microwave power increased, the germination and growth of #2966 spores decreased (Figs. 3(a) and 3(b)), showing that microwave treatment affected both germination and growth. In total, 18 of 60, 15 of 45 and 22 of 41



(a)

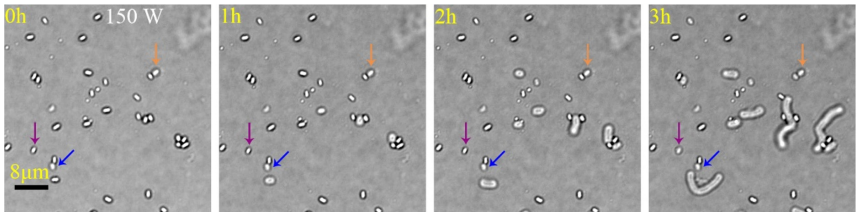


(b)



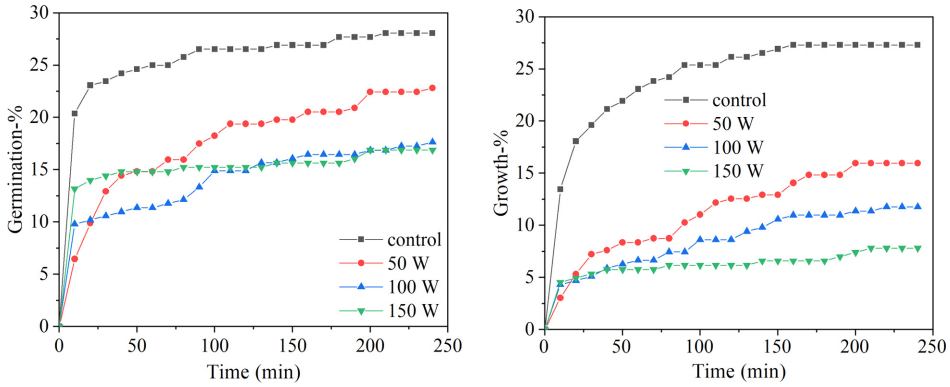
(c)

Fig. 2. Germination, outgrowth and growth of single #2966 spores without or with microwave treatments. (a)–(d) Cell images of untreated and treated group. The blue, orange, and red arrows show the images of spores that germinated immediately. The purple arrows show the images of spores that germinated very late (more than 2 h).



(d)

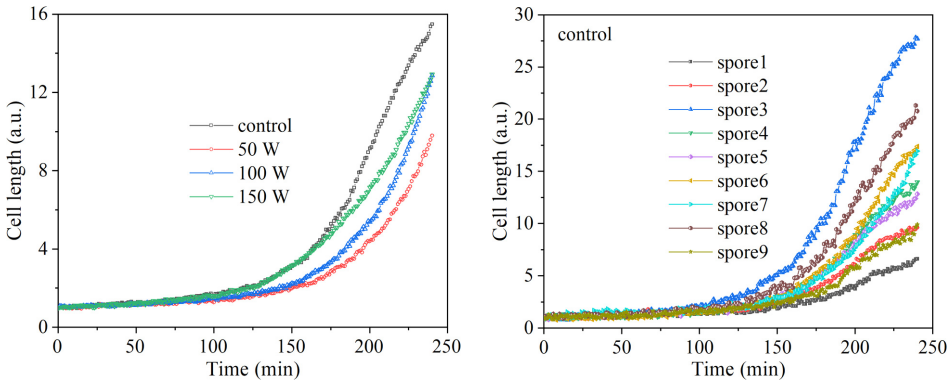
Fig. 2. (*Continued*)



(a) (b)

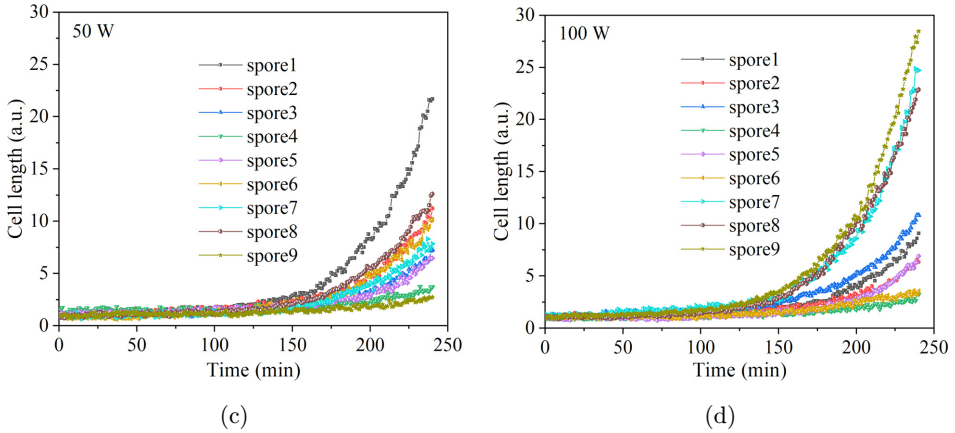
Fig. 3. (a) The percentages of germination and (b) growth of multiple single spores untreated or treated with microwave, ~250 single spores were determined in each microwave condition.

spores germinated but did not grow at 50 W, 100 W and 150 W among ~250 microwave-treated spores counted, compared to only 2 of 73 spores in the untreated group, indicating that microwave treatment at a higher power increased the rate of spores that germinated but did not grow. Furthermore, according to the average spore/cell length versus time curves from nine single spores, we found that cell lengths and the rates of cell lengths decreased (Fig. 4(a)). However, there was variation among



(a) (b)

Fig. 4. (a) Average curves of cell length versus time from nine single spores among powers. (b)–(e) Curves of cell length versus time for nine single spores untreated or already treated at different microwave powers that germinated and grew.



(c) (d)

(e)

Fig. 4. (*Continued*)

individual spores and the cell length versus time curves for nine single spores (untreated or treated) are shown in Figs. 4(b)–4(e). The cell length of spores without microwave treatment was evenly distributed at 4 h and was 6–28 times larger than the original cell lengths. In comparison, 2 of 9, 3 of 9 and 3 of 9 spores showed differences of less than 6-fold at 50 W, 100 W and 150 W, further demonstrating that microwave treatment can affect the growth of spores.

3.3. *Surface morphology of microwaved spores*

To identify the effect of microwave treatments on spore morphology and dimensions, AFM was used to obtain images of spores untreated and treated at 50 W, 100 W and 150 W. Most untreated spores had a relatively smooth outer surface (Fig. 5(a)) and some had shallow ridges on the surface. Microwave-treated spores exhibited relatively prominent shrinking, similar to ridges (Figs. 5(b)–5(d)). Surprisingly, we found that spores treated at 150 W had protuberances covering the whole surface and had a rough appearance (Figs. 5(e) and 5(f)), indicating that microwave treatment affected spore morphology. In addition, as the microwave power increased, the length, width and height of dry spores decreased and the standard deviation of spore length increased substantially, indicating that microwave treatment affects the water content and other internal factors, causing the spores to shrink and higher powers had greater impacts on the volume of spores (Table 2). We also found that average roughness (Ra) along the long axis of spores increased as the power increased, showing that microwave treatment affected the surface roughness of spores in a power-dependent manner.

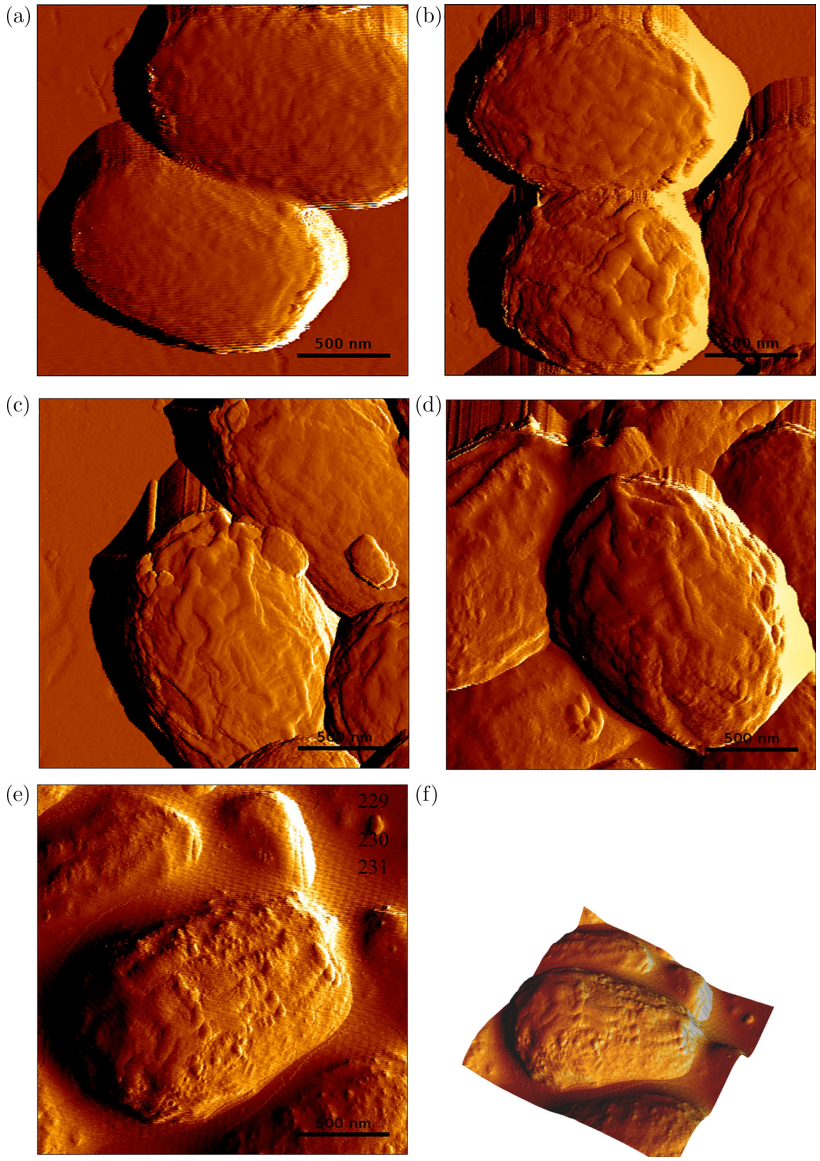


Fig. 5. Without and with microwave treatments spores imaged in air. (a) Amplitude image of spores untreated at a 2 μm scale (scale bar: 500 nm). (b) Amplitude image of spores treated at 50 W power and scanned at a 2 μm scale (scale bar: 500 nm). (c) Amplitude image of spores treated at 100 W power and scanned at a 2 μm scale (scale bar: 500 nm). (d) and (e) Amplitude image of spores treated at 150 W power and scanned at a 2 μm scale (scale bar: 500 nm). (f) 3D height image of spores corresponding to (e).

Table 2. Dimensions of microwaved #2966 spores measured from AFM images.

	Spore length (μm)		Spore width (μm)		Spore height (μm)		Ra[b] (μm)	
	Mean[a]	SD[a]	Mean	SD	Mean	SD	Mean	SD
Control	1.504	0.148	0.981	0.132	0.832	0.116	0.092	0.027
50 W	1.376	0.150	0.950	0.108	0.819	0.207	0.094	0.022
100 W	1.370	0.180	0.942	0.096	0.814	0.121	0.096	0.022
150 W	1.220	0.534	0.939	0.138	0.713	0.154	0.104	0.026

Notes: [a]The mean and standard derivation (SD) values were calculated based on 22 individual measurements on different spores and on separately prepared samples. [b]Average roughness (Ra) along the long axis of spores.

4. Discussion

Previous studies focused on the mechanism for spore killing and have found that microwave radiation at ∼100°C can kill spores. While what we are curious about is the intrinsic effects of microwave on individual spore at single-cell level. This prompted us to examine the effects of microwave radiation on dry spores at room temperature.[34,35] Therefore, we adopted the Discover SP Microwave Synthesizer to investigate the responses of single dry spores to microwave radiation at 25°C. In this investigation, we observed four notable effects on spores: (1) The inactivation of some spores, (2) DNA denaturation, (3) decreases in spore germination, growth and the rate of change in cell length with different microwave powers and (4) obvious morphological changes on the spore surface, depending on the power. These findings raise questions regarding the reason by which microwave treatment at 25°C (i) decreased spore survival, (ii) decreased the rate of growth, (iii) alters cell length changes over time, and (iv) changes spore surface morphology.

It is perhaps not surprising that microwave radiation affects the survival of wet spores, as this is consistent with our results for dry spores.[22,36] Indeed, our findings support the utility of microwave, decreasing survivor of spores. However, the slight change in the CaDPA content indicated that microwave treatment at 25°C for 1 min (i) did not damage the permeability barrier of dormant spores that retain CaDPA, including the inner membrane of the spore, (ii) did not cause changes in molecular structure of CaDPA, but (iii) resulted in molecular changes in the core around CaDPA, maybe by the removal of water inside spores. Previous studies have shown that microwave radiation can damage the inner membrane of spores.[24] In comparison, it did not cause obvious damage to spore structures, like the inner membrane, possibly owing to the short treatment time, low-level power, or low temperature. Therefore, microwave radiation did not destroy spores by damaging the permeability barrier but injured spores via DNA damage. Evidence for this conjecture is as follows: (i) The peak at 782 cm^{-1} due to nucleic acids shifted compared to that for untreated spores, indicating DNA denaturation; (ii) there was no damage to the permeability barrier; (iii) there was no obvious core protein denaturation based on Raman spectra. It is therefore not surprising that some spores germinated but did not grow, as a fully functional intact spore chromosome is not essential for spore germination.[28,35] As the microwave power increased, the proportion of spores that germinated but did not grow increased.

It is normal that spores treated with microwave can still germinate and grow. However, we found that the rate of spore growth decreased and the cell length changed, indicating that microwave radiation could cause abnormal growth. Various factors may explain the effects on growth, including (i) the CaDPA levels and (ii) DNA damage. Of these two alternatives, it seems much more likely that it is caused by DNA denaturation for the following reasons. (i) CaDPA stored at spore core, contributes to dormancy. An increase in CaDPA levels promotes dormancy and therefore decreases the rate of recovery[19] while there were no significant differences in the rate of CaDPA level. (ii) DNA in the spore core contains the genetic information for organismal survival and reproduction, which is crucial for cells to maintain integrity. DNA damage may cause genomic instability and the loss of some functions involved in growth, resulting in abnormal growth.[17,28]

Microwave radiation altered the morphology of spores in particular, it (i) reduced the volume and (ii) increased surface roughness. Most spores had deeply shrunken ridges after exposure to microwave radiation, corresponding to the smaller volume. Additionally, the higher the microwave power, the smaller the volume of the spore. This higher microwave power results in greater effects on the spore with rougher surface. Microwave radiation has thermal effects and penetration ability. After it penetrates the spores, molecules including water interact violently and react with spores.[11,37] In addition, microwave may increase the water mobility throughout the spore, leading to secondary heating to spore. Both can cause the rising of temperature inside spores and then moisture evaporation. To adapt to the space surplus caused by water loss, the multilayer structure shrinks inward, followed by the decrease of spore length and becomes rougher.

5. Conclusions

The most meaningful contribution of this study should be the responses of dry spores to microwave radiation at the single-cell level. We used single-cell

technology to reveal that microwave-treated spores can be inactivated by DNA damage. Surprisingly, the germination rates and rate of spore growth decreased. In addition, the application of atomic force spectroscopy provided a detailed understanding of the surface properties of spores. However, further studies are needed to determine whether microwave radiation at 25°C will damage germinant receptors and the mechanism by which the germination rate decreased with the increase in microwave power.

Conflicts of Interest

The authors have no conflicts of interest relevant to this article.

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