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Effect of Deuterium Oxide on *Bacillus atrophaeus* Spore Germination

CHANG Jinhui[1)*], WU Wen Jie[2,3)*,**]

([1)]*The Hong Kong Polytechnic University Shenzhen Research Institute, Shenzhen* 518052, China;
[2)]*Department of Radiation Physics, The Cancer Hospital of the University of Chinese Academy of Sciences (Zhejiang Cancer Hospital), Institute of Basic Medicine and Cancer (IBMC), Chinese Academy of Sciences, Hangzhou* 310022, China;
[3)]*Zhejiang Key Laboratory of Radiation Oncology, Hangzhou* 310022, China)

Abstract This short communication is to report the inhibitory effect of deuterium oxide (D_2O) on germination and culturability of bacterial spore. Germination was triggered by L-alanine under ambient condition and *in situ* monitored by Tb^{3+}-dipicolinic acid assay, phase contrast microscopy and absorbance measurement. Germination kinetics were characterized by final germination level, germination half time, and germination speed. The culturability of germinated spores was evaluated by enumerating colony-forming units. Results revealed that D_2O retarded the whole germination process and reduced the culturability but didn't affect the final germination level. We postulate that D_2O enhanced the stability of some spore-specific signaling proteins.

Key words deuterium oxide, *Bacillus atrophaeus*, spore, germination, culturability
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Spore formers, including aerobic *Bacillus* and anaerobic *Clostridium*, play a critical role in microbiology with elaborate longevity strategies to withstand environmental upheavals[1]. In the presence of external stress, vegetative cells either die or undergo a process called sporulation to avoid being killed. Spores that formed in sporulation are dormant without detectable metabolic activity[2-3] and are highly resistant to common disinfectants, such as heat, UV irradiation, oxidative agents, chemical toxic agents and desiccation[4]. In spite of dormancy, the resting spores keep on sensing the environment. When favorable conditions arise, they can quickly be revitalized *via* a genetic programme termed as germination and followed by outgrowth. Spores remain safe when they keep on dormancy, but vegetative cells may cause a bunch of food spoilage[5-7], hospital infection[8], human diseases[9], and even bioterrorism[10]. Thus, sporulation and germination are two of the signature physiological activities that create a lot of implications in daily life and lead to numerous applied research interests. In a laboratory setting, germination can be triggered by germinants (a group of specific chemicals). It is also affected by many factors including temperature[11], duration of pre-heat activation[11], pH[12], inoculum size[13], sporulation condition[14], mechanical wave[15] and so on. A recent review with great details on germination can be found at the work of Christie and Setlow[16].

Water is one of the most important small molecules in germination. The content of spore core water is highly correlated with the physiological properties of spore. In dormant spore, the amount of core water is much lower than that in growing cell protoplast, which partially contributes to the resistance of spore to wet heat[17]. In germinating spore, the increased permeability of inner membrane allows external water to flow into spore core. The core pH thus rises from around 6.5 to 7.7[18], which is essential for resuming the activities of many enzymes.

Deuterium oxide (D_2O) has a significant retardant effect on many cellular processes with unclear mechanisms. One of the well-known effects is

*These authors contributed equally to this work.
** Corresponding author.
Tel: 86-571-88202307, E-mail: ee_wwjaa@hotmail.com
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to retard DNA synthesis[19-24]. A possible explanation is that D_2O alters the DNA secondary structures' equilibrium between ordered helix and random coil[25]. Besides, D_2O is also able to lead a "freezing effect" to various types of animal and plant cells[26-29]. A possible mechanism is that D_2O changes the properties of some proteins or polypeptides by replacing their exchangeable hydrogen with deuterium[30]. The resultant effects include increasing their rigidity[31], stability[32-35], and compactness[36], causing denaturation[37], and forming hydration shell around them[38]. Although the permeability of spore inner membrane is very low, ions and small molecules, such as F^- and water, are still exchangeable through it[39-40]. Nevertheless, study on the physiological responses of bacterial spore to deuterium isotope is very limited. Published results indicated that spores prepared in D_2O culture medium had a larger heat resistance and a smaller sporulation rate[41]. Unfortunately, the germination behaviors of spores in D_2O were not examined in those experiments.

This study is to investigate the impact of D_2O, as a substitute for water, on bacterial spore germination. *Bacillus atrophaeus* (*B. atro*) was employed as model species. Germination was triggered by L-alanine. Ca-DPA release was monitored by Tb^{3+}-dipicolinic acid (Tb-DPA) fluorescence assay. Water uptake was tracked by phase contrast microscopy and absorbance measurement. The culturability of spores germinated in D_2O was evaluated by colony-forming units (CFU) enumeration. The properties of D_2O were correlated with the events in germination.

1 Materials and methods

All chemicals, unless otherwise stated, were purchased from Sigma Aldrich (St. Louis MO, USA) and were used without further purification. In addition, all chemicals were sterilized by autoclaving or filtering (0.2-μm filter) prior to use.

1.1 Spore and media preparation

B. atro (ATCC 9372) spore strip was cultured in tryptic soy broth (TSB) (30 g/L) as a general growth medium at 37℃ for 18 h. Exponentially-growing vegetative cells were inoculated into a sporulation medium, which consisted of 1.6% nutrient broth, 1.5% agar, 0.2% KCl and 0.05% $MgSO_4$, 1 mmol/L $Ca(NO_3)_2$•$4H_2O$, 100 μmol/L $MnCl_2$•$4H_2O$, 1 μmol/L $FeSO_4$•$7H_2O$, and 0.1% glucose. The cells were incubated for 4 d at 37℃ followed by an overnight lysozyme (500 μg/L, 0.2-μm-filter-sterilized) digestion at 37℃. Spores were then harvested and purified by 10 times of centrifugation at 11 180 *g* at 4℃ and washed until a 99.9% purity was reached as verified by haemocytometer (Petroff-Hausser, Horsham PA, USA) counting under an Eclipse Ti-U inverted phase contrast microscope (Nikon, Japan). Stocks of spore suspension were stored at 4℃ refrigerator in the dark[42].

1.2 Germination study

Unless otherwise noted, all germination experiments were run in triplicate at (25 ± 2)℃. The solvent of experiment group was pure D_2O and the control experiments (*i. e.*, H_2O-based germination) were conducted as appropriate.

1.2.1 Tb-DPA fluorescence assay

Tb-DPA fluorescence assay was employed to monitor the DPA release process during germination of bulk spore suspension. 20-μl water suspension of spore (2×10^{12} cells/L) was added to a PMMA cuvette (Dynalon, Rochester NY, USA) and air-dried at 0℃, followed by adding equal amount of D_2O or H_2O as sample or control group. Germination media were then prepared to a final volume of 2 ml containing 2×10^{10} spores/L spore, 10 mmol/L L-alanine, 100 μmol/L $TbCl_3$, and buffered in 50 mmol/L Tris-HCl at pH= 7.6. Emission spectra (λ_{ex} = 278 nm, λ_{em} = 450 nm − 560 nm) and excitation spectra (λ_{ex} = 250 nm − 360 nm, λ_{em} = 545 nm) were measured by Fluorolog-3 fluorometer (Horiba Jobin Yvon, Edison NJ, USA) against time. The fluorescence signal was recorded as $I_{D_2O}(t)$. After 2-hour germination, medium was autoclaved such that all spores were ruptured and all DPA was released. Medium was scanned again, and the intensity were recorded as $I_{D_2O\_total}$. Time course of germination ratio was denoted as $r_{D_2O\_Tb-DPA}(t)$ and quantified by

$$r_{D_2O\_Tb-DPA}(t)=\frac{I_{D_2O}(t)}{I_{D_2O\_total}} \quad (1)$$

The data of control group was denoted as $r_{H_2O\_Tb-DPA}(t)$.

1.2.2 Phase contrast microscopy

Change of individual spore's refractility during germination was caused by water intake and can be monitored by phase contrast microscopy. A 2-μl

droplet of spore suspension (2×10^{11} spores/L) was dropped on a microscope slide (Marienfeld, Germany), carefully spread, and air-dried for around 5 min. After gentle washing to remove unattached spores, a 5-μl droplet, which contained 10 mmol/L L-alanine and was buffered in 50 mmol/L Tris-HCl at pH =7.6, was dropped on the dried pattern of spore and covered by a coverslip (Marienfeld, Germany). The edges of coverslip were then sealed by a thick layer of nail polish to minimize water evaporation. The slide was observed under the inverted phase contrast microscopy. Images were taken with a CCD camera (SPOT Xplorer, Nikon, Japan) against time. Each field of view contained (100±20) spores. Germination was quantified by calculating the ratio of phase dark body to the total number of spores in that view and was recorded as $r_{D_2O\_PC}(t)$. The data of control group was denoted as $r_{H_2O\_PC}(t)$.

1.2.3 Absorbance measurement

Uptake of water into spore core during germination leads to refractility decrease. This process was measured by loss of absorbance at 600 nm (A_{600}). Germination media were prepared in 96-well transparent polystyrene plates (SPL Life Sciences, South Korea) to a final volume of 200 μl containing 2×10^{12} spores/L spore, 10 mmol/L L-alanine and 50 mmol/L Tris-HCl buffer at pH=7.6. A_{600} was measured by an Elx800 absorbance microplate reader (BioTek, Winooski VT, USA) against time and recorded as $A_{600\_D_2O}(t)$. Before each reading, spore suspensions were uniformed by pipetting up and down. Time course of germination ratio was then plotted as

$$r_{D_2O\_A}(t) = 1 - \frac{A_{600_{D_2O}}(t)}{A_{600_{D_2O}}(0)} \tag{2}$$

The data of control group was plotted as $r_{H_2O\_A}(t)$.

1.2.4 Culturability test

CFU enumeration was used to evaluate the culturability of spores germinated in D_2O or H_2O media. Spores (2×10^{10} cells/L) germination was triggered by 10 mmol/L L-alanine buffered in 50 mmol/L Tris-HCl at pH=7.6. After 2 h, germinated spore was diluted by H_2O (for both sample and control group) to 2×10^6 cells/L. 100 μl suspension was then transferred on tryptic soy agar (TSA) and inoculated at 37℃ for outgrowth and vegetative growth. TSA was prepared in H_2O (for both sample and control group) and contained 15 g/L agar (Oriental Chemicals & Lab. supplies Ltd., Hong Kong) and 30 g/L TSB. CFU number was enumerated after 12 h. Culturability ratio was defined as the number of CFU divided by the total number of spores inoculated.

2 Results

The cumulative form of the Weibull distribution[43] was used to fit data points from each germination experiment:

$$P(t) = P_{asym}\left\{1 - \exp\left[-\left(\frac{t}{t_c}\right)^m\right]\right\} \tag{3}$$

where $P(t)$ is the germination ratio against time, P_{asym} is the asymptotic value and used as the final germination level. Besides, we define germination half time $T_{1/2}$ as the time when $P(t)$ reaches the half value of P_{asym},

$$P(T_{1/2}) = \frac{1}{2}P_{asym} \tag{4}$$

and germination speed k as the slop of tangent line of $P(t)$ at $t = T_{1/2}$:

$$k = \left.\frac{dP(t)}{dt}\right|_{t=T_{1/2}} \tag{5}$$

From Equation (3)−(5) we can obtain

$$T_{1/2} = t_c(\ln 2)^{\frac{1}{m}} \tag{6}$$

$$k = \frac{P_{asym}m}{2t_c}(\ln 2)^{\frac{m-1}{m}} \tag{7}$$

External factor decreases final germination level if P_{asym} decreases, retard germination process if $T_{1/2}$ increases or k decreases[43].

Unpaired student's t-test was used to analyze differences of parameters (P_{asym}, $T_{1/2}$, k and culturability), using MATLAB® statistics software. The difference was considered as significant if $P < 0.05$. Percentage change of P_{asym}, $T_{1/2}$, k and culturability between sample and control were expressed as (sample – control)/control×100%.

In Tb-DPA fluorescence assay, the germination time course (Figure 1a) and percentage difference (Figure 2) showed that germination in D_2O increased $T_{1/2}$ by 16%, but did not significantly affect P_{asym} and k ($P > 0.05$). In phase contrast microscopy, the germination time course (Figure 1b) and percentage difference (Figure 2) showed that D_2O increased $T_{1/2}$

by 49.5% and decreased k by 31.1%, but did not significantly affect P_{asym} ($P > 0.05$). In A_{600} measurement, the germination time course (Figure 1c) and percentage difference (Figure 2) showed that D_2O increased $T_{1/2}$ by 69.3% and decreased k by 33.8%, but did not significantly affect P_{asym} ($P > 0.05$). All of these three methods have consistent conclusion that D_2O did not affect final germination level but retarded the germination process. Furthermore, D_2O decreased culturability by 68.3% (Figure 2).

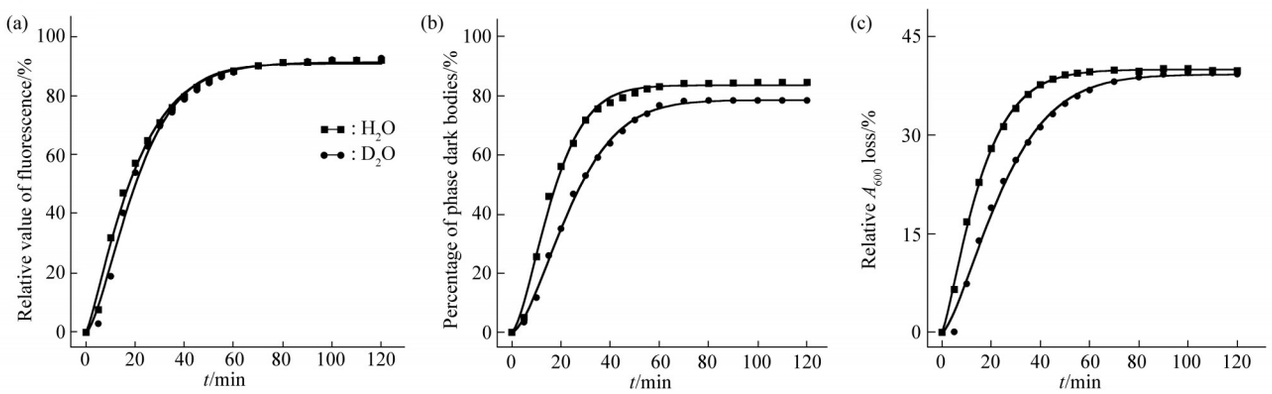

Fig. 1 Kinetics of germination of a spore population

Spores of *B. atro* were germinated with L-alanine in H_2O (■) or D_2O (●) as described in Materials and Methods. The detection method was Tb-DPA fluorescence assay (a), phase contrast microscopy (b) or A_{600} measurement (c). Data were fitted by Equation (4).

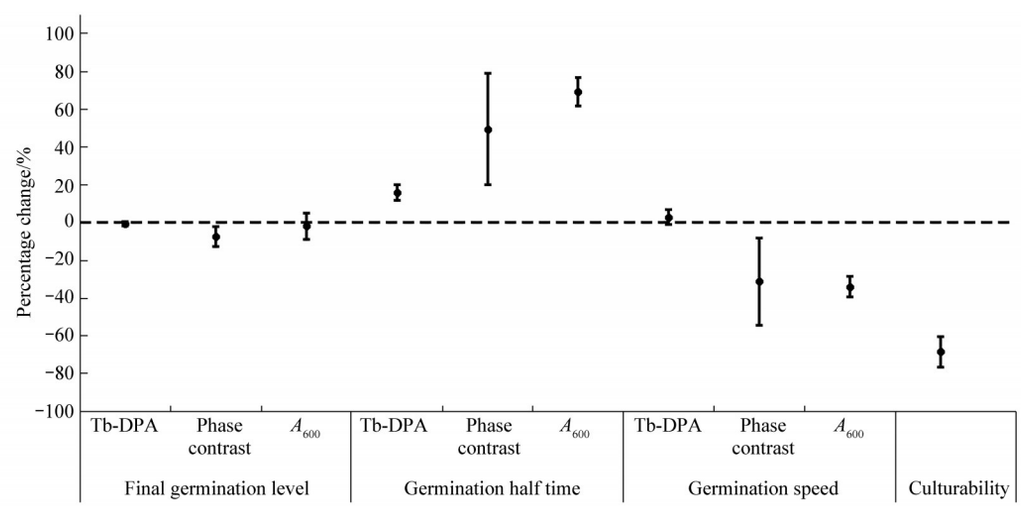

Fig. 2 Percentage changes of germination parameters between *B. atro* spores germinated in H_2O or D_2O

Percentage changes were expressed as (sample − control)/control×100%.

3 Discussion

Different isotopes of an element always share similar chemical properties, but the case is quite different for hydrogen. The atomic mass of deuterium is approximately 100% higher than that of hydrogen. When the hydrogen in H_2O is replaced by deuterium, a lot of physical and chemical properties, including density, dynamic viscosity, attenuation coefficient and hydrogen bond energy, are changed. These changes may influence biosynthetic and cellular reactions of living organisms.

One of the well-known effects of D_2O is to enhance the stability of protein[32-33, 44]. During germination, various biophysical processes such as response of germination receptors (GRs) to germinants and regulation of Ca-DPA release, are precisely mediated by respective proteins. Take GR as

an example, it is a spore inner membrane (IM) protein that recognizes specific germinants and triggers subsequent germination events. Rather than uniformly or randomly distributed among spore's IM, GRs are presented together with GerD proteins to form multiple clusters, termed as germinosomes[45]. Recent work indicated that one or more germinosome proteins in spores undergo some changes in state soon after germinant addition[46-47]. Therefore, the retarded germination observed in our study was not a surprise, and it is possible that the enhanced stability of germinosome in D_2O makes a hindrance to its conformational changes in response to germinant. Besides GRs and GerD, some other important proteins in germination includes GerP and SpoVA. GerP is to facilitate the access of germinants to their cognate GRs and spore with a *gerP* deletion mutation has slower germination speed[48-49]. SpoVA proteins are involved in DPA uptake during sporulation[50] and Ca-DPA release during germination[51]. They make up at least part of the Ca-DPA release channels[52] but their exact role, such as how to gate Ca-DPA release, is not clear. The possibly enhenced stability of GerP and/or SpoVA may also slow down germination.

Suppression of DNA synthesis is another effect that D_2O exerts. Results also showed that D_2O reduced spore's culturability. DNA of dormant spore is bound and saturated with a group of α/β-type small, acid-soluble proteins (SASPs), which alters the DNA conformation to protect spore from being inactivated by UV irradiation. In a SASP-DNA complex, DNA directly contacts with the carboxyl-terminal region of α/β-type SASP[53] to form chemical bonds, probably hydrogen bonds[54]. During germination, α/β-type SASPs are dissociated from DNA and eventually degraded to amino acids by germination protease. Uncompleted dissociation leads to a lower culturability of germinated spores[55], but failure to degrade free α/β-type SASP does not have such effects[56]. Thus, even if the stability of α/β-type SASP was increased by D_2O, it is unlikely to cause a lower culturability. Change of DNA's secondary structure by D_2O is also unlikely the reason since the transition between ordered helix and random coil is reversible. In sample group, H_2O was fully available when the germinated spore was transferred to TSA for outgrowth and vegetative growth, while a lower CFU number was still observed after a sufficiently long incubation time. A more probable explanation is that replace of hydrogen by deuterium may hamper the dissociation process by increasing the bond energy between α/β-type SASP and DNA.

Consequently, D_2O retards germination, probably by enhancing the stability of some spore-specific proteins. In addition, D_2O possibly prevent the dissociation between spore's DNA and α/β-type SASP, causing a lower culturability. Identify which biomolecules are exactly affected and how they are affected by D_2O are directions of future investigation. Also, to ensure the generality of results, other *Bacillus* species should be studied in future work.

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重水对萎缩芽孢杆菌芽孢萌发的影响

常金辉[1)*]　吴文捷[2,3)*,**]

([1)] 香港理工大学深圳研究院，深圳 518052；

[2)] 中国科学院大学附属肿瘤医院（浙江省肿瘤医院），中国科学院基础医学与肿瘤研究所，杭州 310022；

[3)] 浙江省放射肿瘤学重点实验室，杭州 310022）

摘要　本研究快报报道了重水对细菌芽孢的萌发及其可培养性的抑制作用. 在常温条件下，用L-丙氨酸触发细菌芽孢的萌发，并用Tb-DPA荧光法、相差显微镜观测法和光密度测定法监测萌发过程，用最终萌发水平、萌发半期、萌发速度3个参数来表征萌发过程. 除此之外，我们还用菌落形成单位的个数来评估萌发后芽孢的可培养性. 结果表明，重水对整个萌发过程有抑制作用，同时降低了萌发后芽孢的可培养性，但对最终萌发水平无影响. 我们推测这是因为重水增强了一些芽孢特异性信号蛋白的稳定性.

关键词　重水，萎缩芽孢杆菌，芽孢，萌发，可培养性

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* 并列第一作者.

** 通讯作者.

Tel：0571-88202307，E-mail：ee_wwjaa@hotmail.com

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