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Full Length Research Paper

Isotope effects on cell growth and sporulation, and spore heat resistance, survival and spontaneous mutation of *Bacillus cereus* by deuterium oxide culture

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Deuterated spores of *Bacillus cereus* were prepared using a multi-stage adaptation protocol and then preserved at -20, 4, 25 and 40°C, respectively, to investigate the genetic mutation effects of deuterium oxide (D_2O) isotope. The effects of the D_2O isotope on cell growth and sporulation, and spore heat resistance, survival and spontaneous mutation rate of spores were examined. The results suggested that *B. cereus* cells can be grown in a cultured medium containing 98% D_2O, with no observed growth inhibitory effect. The improved heat resistance of the deuterated spores and the decreased sporulation rate were accompanied by an increase in the D_2O concentration in the cultured medium. Although, the survival rate of spores reduced along with the extension of the preservation time, in contrast to spores cultured by H_2O medium, the deuterated spores had higher survival rate at -20 and 40°C at storage, respectively. In general, the spontaneous mutation rate increased along with preservation temperature and time. The spontaneous mutation rate of the deuterated spores significantly decreased when compared with that of hydrogen culture ($P<0.05$). This result provided a preliminary experimental evidence to validate the Löwdin DNA mutation model based on the inter-base double proton transfer in a hydrogen pond of base pairs.

Key words: Deuterium oxide, Isotope effect, spontaneous mutation, proton-tunneling model.

INTRODUCTION

The special significance of deuterium in biological systems has been recognized shortly after its discovery by Urey in 1932. A considerable range of living organisms have been cultivated on media containing deuterium oxide (D_2O) since the 1960s (de Giovanni, 1960). The isotopic effect of D_2O on cell growth and cell division has attracted the interest of previous researchers. Significant differences in the morphology and biosynthetic capacity between the hydrogen-containing organisms and the deuterated organisms have been observed (Flaumenhaft et al., 1965). Intriguing results have been reported, such as the use of heavy isotopes to avert aging (Demidov, 2007), D_2O anticancer effect on bladder cancer cell (Bahk et al., 2007), metabolic switching due to deuterated drug, where the changed metabolism may lead to increased duration of action and lower toxicity (Kushner et al., 1999) and so on. However, whether D_2O has a deuterium isotope effect

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on genetic mutation is an issue that has been largely overlooked by biologists. DNA mutation is a fundamental and important biological process, which not only underlies the evolution of life but also induces many diseases, such as cancer and inherited diseases. Thus, the elucidation of the molecular mechanisms of DNA mutation is of great significance. Considering the ability of spores to survive for long periods without nutrients, as well as their metabolic dormancy, the possibility of well-known erroneous replication mechanism can be excluded simultaneously. Therefore, spores are very suitable for investigating the mechanism of spontaneous mutation accumulated during the period of dormancy. In the present paper, deuterated spores of *Bacillus cereus* were prepared in a cultured medium containing 98% D_2O. The D_2O isotope effects on cell growth, sporulation, and heat resistance of spores were investigated. Deuterated spores and normal hydrogen-containing spores were preserved at different temperatures. The survival rate and the spontaneous mutation rate of the spores were examined at storage to deter-mine the isotope effect of D_2O culture on genetic mutation.

MATERIALS AND METHODS

Bacteria strains and medium

The ciprofloxacin (CPLX)-sensitive *B. cereus* strain CMCC (B) 63301 was obtained from the Chinese National Institutes for Food and Drug Control. Luria–Bertani (LB) liquid medium and LB agar medium were used to grow this bacterium and obtain a viable colony count. For the preparation of LB/CPLX agar medium, 0.1 mg/mL CPLX in LB agar medium was used. Sporulation Y1 medium (de Vries et al., 2004) was a chemically defined medium which contained the final concentrations of the following: D-glucose (10 mmol/L), L-glutamic acid (20 mmol/L), L-leucine (6 mmol/L), L-valine (2.6 mmol/L), L-threonine (1.4 mmol/L), L-methionine (0.47 mmol/L), L-histidine (0.32 mmol/L), sodium-DL-lactate (5 mmol/L), acetic acid (1 mmol/L), $FeCl_3$ (50 µmol/L), $CuCl_2$ (2.5 µmol/L), $ZnCl_2$ (12.5 µmol/L), $MnSO_4$ (66 µmol/L), $MgCl_2$ (1 mmol/L), $(NH_4)_2SO_4$ (5 mmol/L), Na_2MoO_4 (2.5 µmol/L), $CoCl_2$ (2.5 µmol/L) and $Ca(NO_3)_2$ (1 mmol/L). The medium was buffered at pH 7.2 with 100 mmol/L potassium phosphate buffer made up of distilled H_2O and 25, 50, 75 and 98% D_2O, respectively.

Adaptation of D_2O culture

B. cereus strains were adapted to grow on deuterated Y1 medium by repeated subculturing, gradually increasing the deuterium concentration of Y1 medium from 25 to 98%. At first, the *B. cereus* strains were streaked on LB agar plate and grown overnight at 30°C. Single colonies were selected for shaking culture in LB liquid medium at 30°C for 12 h. Subsequently, all cell cultures were initiated by inoculation with 1% of the sample from the previous culture up to an A_{600} of 0.1 and were grown to stationary phase. The culture was grown successively on Y1 medium made up of distilled H_2O and 25, 50, 75 and 98% D_2O, respectively. During the adaptation of D_2O culture, the A_{600} of a 5× diluted culture was measured at regular time intervals from 0 to 48 h to investigate the isotope effect of D_2O culture on cell growth.

Sporulation rate assay

During adaptation of the D_2O culture, the sporulation process was monitored using Albert's stain method, while the cell entered the stationary phase. After 60 to 72 h incubation, the sporulation efficiency was counted as spores/total viable counts × 100%. All experiments were performed in triplicate.

Heat resistance assay

A total of 100 µl spore suspension was sealed on the tip of a Pasteur pipette. The Pasteur pipette was heated at 90°C by total immersion in a thermostatically controlled water bath. At appropriate time intervals, the Pasteur pipette was removed and immediately cooled in an ice-water mixture. Serial dilution was made in 10 mmol/L potassium phosphate buffer (pH 7.4) and plated onto LB agar. The colonies were then counted after 2 days of incubation at 30°C (de Vries et al., 2005). The survival curves were plotted as $\log_{10}$ of the surviving organisms against time. The decimal reduction time (D value), which is the time required to reduce the number of surviving organisms by 90% at a constant temperature, was calculated from the regression analysis of $\log_{10}$ of the survivor for various time intervals using Origin software (version 7.0 for Windows XP).

Preparation and preservation of spores

After 48 h incubation in H_2O medium or 72 h incubation in 98% D_2O medium, the spores were harvested by centrifugation (10,000 ×g) for 30 min. The pellet was suspended in 10 mmol/L potassium phosphate buffer (pH 7.4) with 0.1% Tween 80. The vegetative cells and cell debris were removed from the suspension by washing five times. Separation was conducted by differential centrifugation (600 and 2500 ×g). The prepared spores were stored at 4°C for later use (Mazas et al., 1995). The spore suspensions were adjusted to approximately 3.0×10^9/ml and 0.2 ml spore suspension was added in each preserving sand tube. After vacuum freeze drying and sealing, the preserving sand tubes were stored at -20, 4, 25 and 40°C, respectively.

The effect of D_2O culture in survival rate and spontaneous mutation rate

The spontaneous mutation rate was measured on the 1st, 90th, 180th and 360th days during storage. Each preserving sand tube was washed three times with 10 ml distilled water by centrifugation at 1200 ×g for 3 min. The supernatant was pooled with a distilled water volume of 10 ml. A total of 100 µl of the supernatant was sampled to analyze the survival rate by viable colony count. The survival rate was calculated using the 1st day samples at 100% level. The remaining supernatant was filtered through a 0.22 µm filter membrane and plated onto an LB/CPLX agar plate. The mutation of the antibiotic-resistant colonies was counted after overnight incubation at 30°C. Each treatment was carried out with three replicates. The spontaneous mutation rate of the spore was calculated using the quotient of the number of antibiotic-resistant colonies and the survivors of the preserving sand tube.

Statistical analysis

Statistical analyses were carried out using SPSS software (version 17.0 for windows XP). The results were expressed as the mean va-

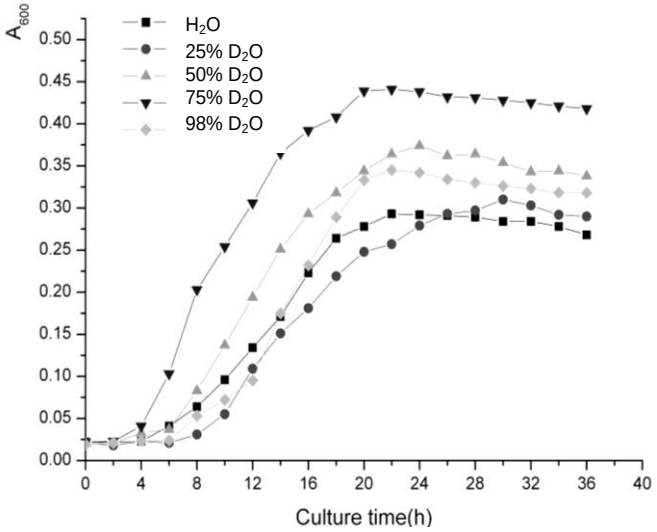

Figure 1. The growth curve of *B. cereus* during the adaptation to 98% D_2O medium.

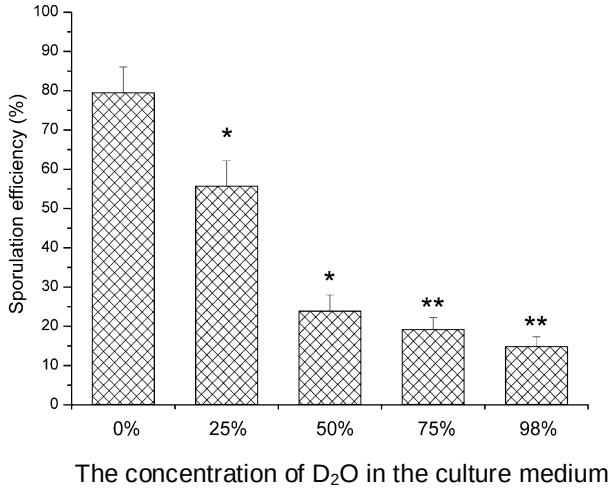

Figure 2. The isotope effect of D_2O culture in the sporulation of *B. cereus.* *Represents significant difference between the different concentrations of the D_2O medium and 0% as control ($P < 0.05$) and **represents very significant difference ($P < 0.01$).

lue ± SEM (statistical error means). A post hoc analysis was performed using Tukey's post test. The survival rate data and the spontaneous mutation rate were analyzed using one-way ANOVA. Student's *t*-test was used for additional pairwise comparisons.

RESULTS

The isotope effect of D_2O culture on cell growth

The growth curves showed the ability of *B. cereus* to grow in Y1 medium containing different concentrations of D_2O (Figure 1). The growth of *B. cereus* was inhibited by Y1 medium containing 25% D_2O when compared with full H_2O medium. A prolonged lag phase and a delayed stationary phase were observed. However, the growth of *B. cereus* was stimulated by D_2O medium ranging from 50 to 98% in contrast to H_2O medium during successive adaptation. Furthermore, decreased growth was observed between 75 and 98% D_2O medium.

The isotope effect of D_2O culture on sporulation

The periodic microscopic examinations of the stained smears revealed that the sporulation process was slowed by the D_2O medium. The appearance of spores cultured in the medium containing different concentrations of D_2O was deferred to 60 h, whereas spores formed after 24 h incubation in H_2O medium (data not shown). Furthermore, a significant difference between the D_2O and H_2O culture (Figure 2) was observed. The sporulation efficiency was reduced from 79.5 to 14.83% with the increase in the concentration of D_2O in the medium from 0 to 98%. One-way ANOVA proved that the effect of the concentration of D_2O in sporulation was significant ($P < 0.05$). Pairwise comparisons between the different concentrations of D_2O medium showed that there was no significant difference among the treatments under 50% D_2O levels.

The isotope effect of D_2O culture on heat resistance of spores

The heat resistance property of spores can be assayed using the D_{90} value obtained from the reciprocal of the slope of the regression line (Figure 3). The increase in heat resistance (D_{90} value) was observed when the spores were prepared by D_2O medium. The increased heat resistance levels varied among the different concentrations of D_2O in the medium and ranged from 227.27 min for the D_{90} value of 75% D_2O medium to 33.69 min for the D_{90} value of 25% D_2O medium. Except for the 25% D_2O medium, the heat resistance levels (D values) of the spores were significantly higher than that of the spores cultured by H_2O medium ($P < 0.05$).

The isotope effect of D_2O culture on the survival rate of spores at storage

The isotope effect of D_2O culture in the survival rate of spores at different preservative temperatures as assessed at interval periods during storage is shown in Figure 4. The analysis of the results showed that both 98% D_2O and H_2O culture had significant difference in terms of preservative time, preservative temperature and survival rates of spores during storage ($P < 0.05$). The maximum

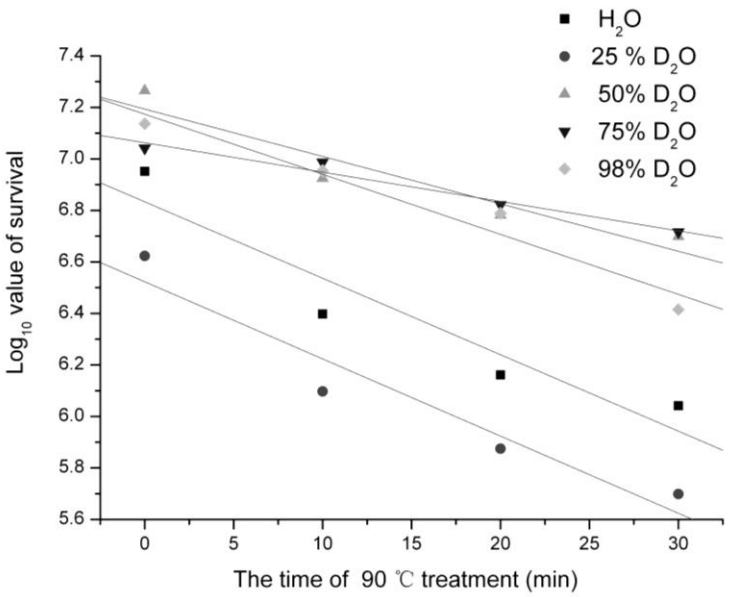

Figure 3. The isotope effect of D_2O culture in the heat resistance of *B. cereus* spores.

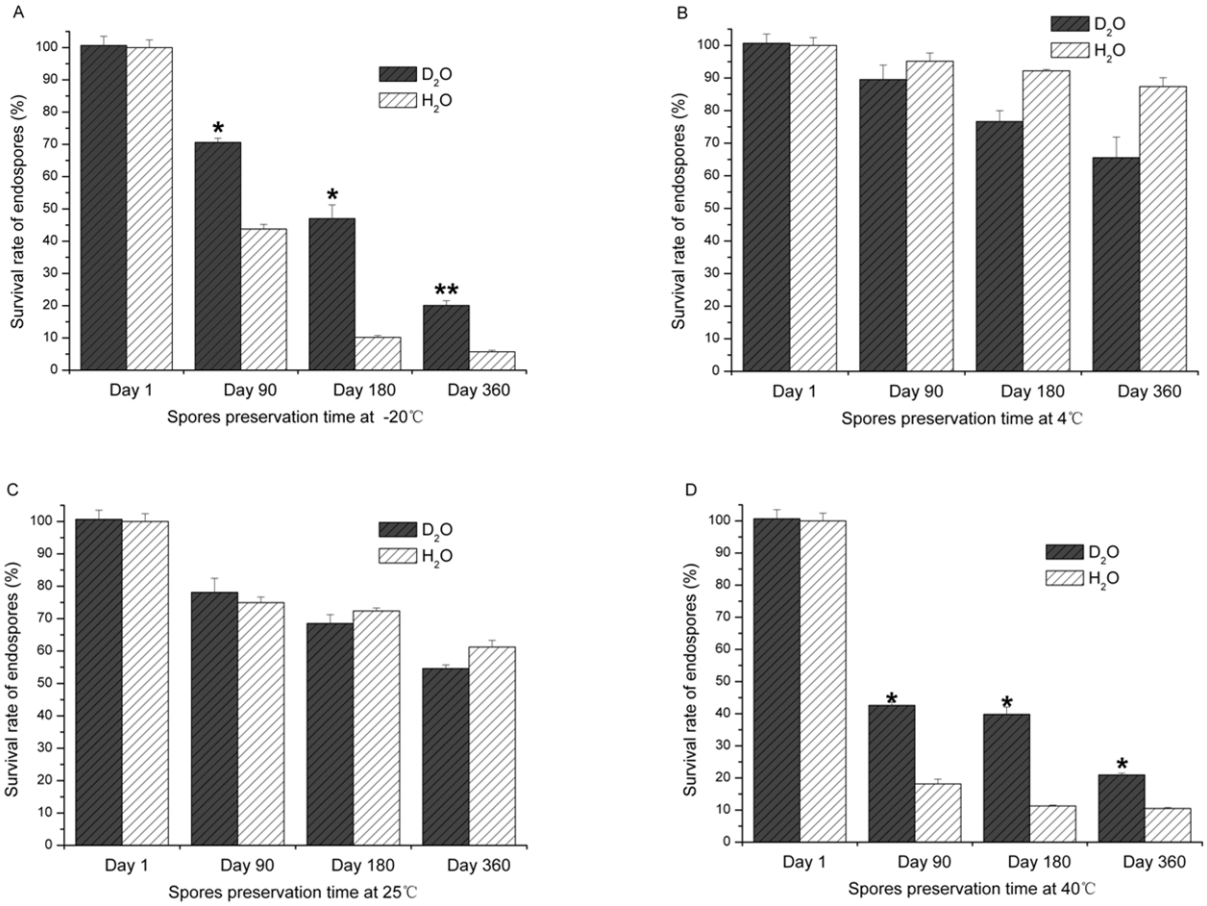

Figure 4. The isotope effect of D_2O culture in the survival rate of spores at storage. *Represents significant difference between D_2O and H_2O cultures ($P < 0.05$) and **represents very significant difference ($P < 0.01$).

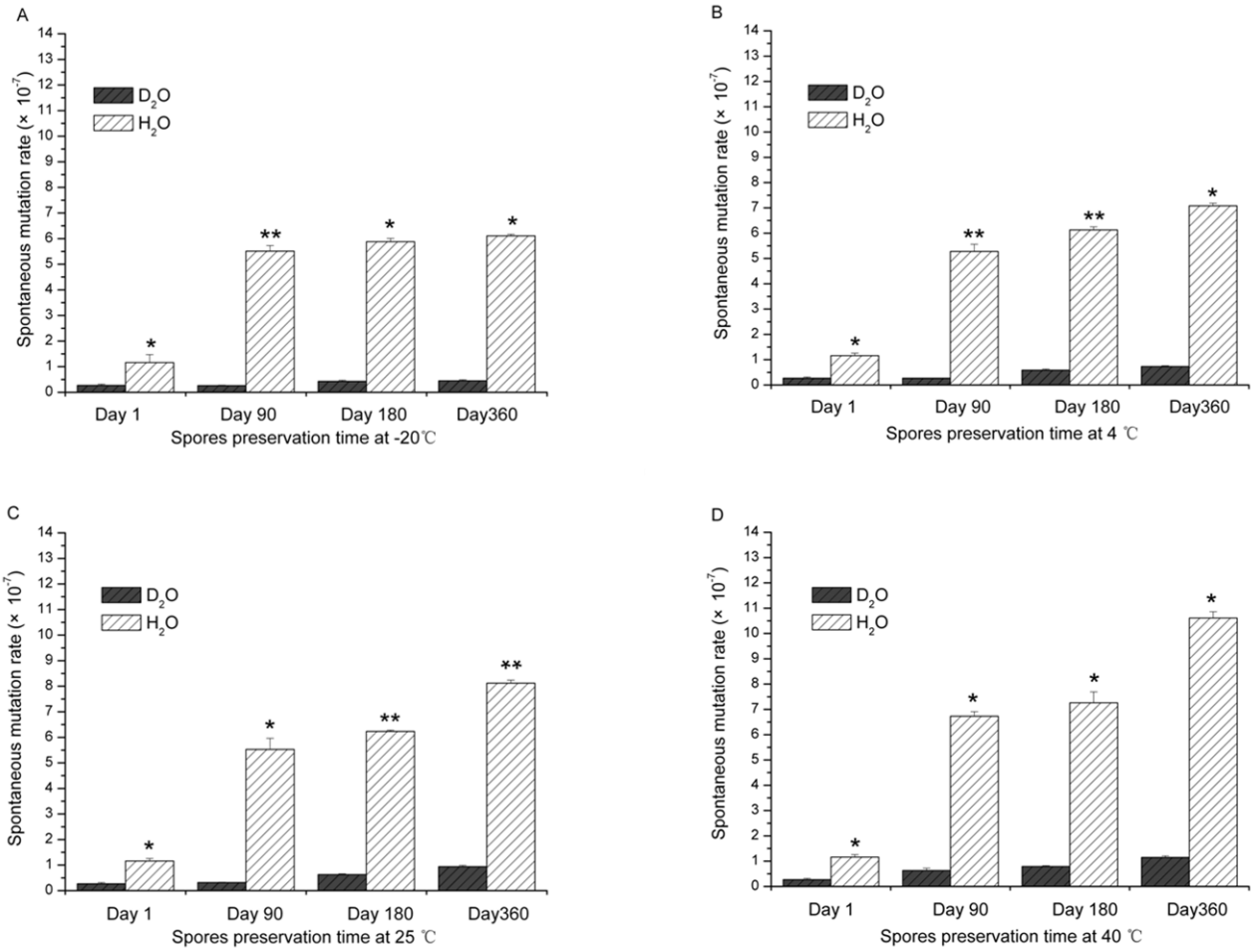

Figure 5. The isotope effect of D_2O culture on the spontaneous mutation rate of spores during storage. *Represents significant difference between D_2O and H_2O cultures ($P < 0.05$) and **represents very significant difference ($P < 0.01$).

survival rate in both D_2O and H_2O medium was obtained at 4°C after 360 days. Significant difference in the survival rate was not observed between 25 and 4°C. Moreover, the *t*-test result showed no significant difference in the survival rate of spores in the D_2O and H_2O culture at 4 and 25°C during all periods of storage. At -20 and 40°C, the survival rates of spores cultured by D_2O medium were reduced to 20.14 and 20.19% at 360 days, respectively. The survival rates of spores cultured by H_2O medium were also reduced to 5.7 and 10.49% at 360 days, respectively. Furthermore, the *t*-test result showed that the D_2O culture had a significantly increased survival rate when compared with the H_2O culture at -20 and 40°C, respectively.

The isotope effect of D_2O culture on spontaneous mutation

The isotope effect of D_2O culture on spontaneous mutation rate is shown in Figure 5. The spontaneous mutation rate was significantly decreased after adaptation to 98% D_2O medium when compared with that of H_2O culture ($P < 0.05$). The spontaneous mutation rates were significantly increased with preservation time ($P < 0.05$). For the D_2O culture, the spontaneous mutation rates increased from 0.27 ± 0.05 ($\times 10^{-7}$) to 0.45 ± 0.04, 0.73 ± 0.09, 0.94 ± 0.05 and 1.15 ± 0.06 ($\times 10^{-7}$) when spores were preserved at −20, 4, 25 and 40°C, respectively. As for the H_2O culture, the spontaneous mutation rate increased from 1.16 ± 0.10 ($\times 10^{-7}$) to 6.11 ± 0.06, 7.08 ± 0.34, 8.12 ± 0.12 and 10.61 ± 0.25 ($\times 10^{-7}$), respectively. In any test time, the spontaneous mutation rate increased with higher temperature. However, a significantly reduced mutation rate was observed between D_2O culture and H_2O culture.

DISCUSSION

Adaptation to D_2O medium

The substitution of hydrogen by deuterium in higher plants

and animals was, in general, a toxic process, whereas deuterium was less toxic for the unicellular forms of life when compared with the higher forms of organisms. A previous study proved that the growth of *Staphylococcus albus* is not inhibited by D_2O concentrations as high as 92% (Lester et al., 1960). The growth of several strains of bacteria has been reported to be inhibited by the D_2O medium, but the degree of inhibition is strain-specific, in addition to the incorporation of 0.5% NaCl (w/v) in the D_2O medium, which decreases the inhibition to bacteria growth (de Giovanni, 1960). A multi-stage adaptation protocol has been improved for the cultivation of *Escherichia coli* in a fully deuterated medium (Paliy et al., 2003). In the current research, the growth of *B. cereus* was stimulated by 50 and 75% D_2O medium, thus validating the universality of the multi-stage adaptation protocol. The greatly reduced growth in the 98% D_2O medium can be attributed to the toxicity of high concentrations of D_2O.

The isotope effect of D_2O culture in the sporulation of *B. cereus*

Spore formation is the ultimate example of bacterial adaptation to starvation. Unlike most bacterial adaptive responses, sporulation takes many hours and includes major changes in the cellular morphology, biochemistry and physiology. The process can be divided into five major phases, four of which are coordinated by sporulation-specific sigma factors, which act as master regulator genes, effectively determining the cell type at any given stage (Setlow, 2006). Once the appropriate intrinsic and extrinsic conditions for sporulation have been met, the initiation of sporulation occurs. This process involves a modified, highly specialized cell division resulting in a series of morphological changes different from those that occur in the vegetative cells. These events are directed, at least in part, by changes in the transcriptional apparatus of the cell (Xenopoulos and Piggot, 2011; Saujet et al., 2011). The changes in the enzyme reaction rates and metabolic pathway were due to the incorporation of deuterium into the substrates or enzymes of *E. coli* (Hochuli et al., 2000; Zhang et al., 2009). The decreased sporulation after growth in the deuterated medium was likely a result of a metabolism and transcription disturbance. The hydrogen-stable isotope ratios of organic matter were linearly related to those of the culture medium (Kreuzer-Martin et al., 2003). The adaptation of *B. cereus* was performed in a 98% D_2O medium to obtain highly deuterated spores.

Heat resistance of spores

The wet heat resistance factors included dehydration of the spore core, protection of the spore DNA by small acid soluble proteins (SASP), DPA (pyridine-2, 6-dicarboxyic acid) and accumulation of divalent cations (Setlow, 1995). The major factor that determined the wet heat resistance of the spore was core water content. There was an inverse relationship between core water content and wet heat resistance. The slight difference between 25% D_2O culture and normal water culture can be ascribed to the unadapted cells that took up H_2O from the growth medium in preference to D_2O. Instead of a progressive increase in the concentration of D_2O in the cell during adaptation, the condition exhibited a similar dehydration effect because D_2O has a higher density (1.105 g/cm^3) when compared with H_2O. The poorly hydrated proteins are generally more heat resistant than proteins in a solution because of the reduced molecular motion. Previous studies have clarified that D_2O could enhance the thermostability of some virus. The hydrogen-deuterium exchange occurred in the viral particles, and RNA structure was stabilized as a result of the D_2O-treatment. Furthermore, the increased δD_{SMOW} of D_2O-treated virus correlated well with its enhanced thermostability (Wu et al., 1995; Sen et al., 2009). Thus, the stability of the deuterated macromolecule presumably played a role in the heat resistance of deuterated spore.

Survival rate of spores during storage

High concentration of D_2O causes toxic effects on a number of biological systems. However, the experiments designed to study the effect of D_2O on bacteria starvation in buffer solutions showed that D_2O is not toxic under these conditions. Furthermore, longevity of the bacteria increases (Lovett, 1964). This result proves that D_2O retards RNA hydrolysis rate, which is partly used as an energy source in maintaining the viability of the organism. Spores preserved at 40°C were subjected to dry heat rather than wet heat. In contrast to spore death by wet heat, the death of wild-type spores by dry heat was accompanied by the accumulation of DNA damage. Presently, the only factor that has been shown to contribute to spore dry heat resistance is the protection of spore DNA by the α/β-type SASP. The binding of the α/β-type SASP to spore DNA, coupled with spore core dehydration, appears to change the helical conformation of spore DNA from B form to A-like form (Nicholson et al., 1990; Setlow, 1992). Considering that D_2O can have a similar dehydration effect in the spore cells, the increased survival rate appears reasonable. In addition, the presence of a neutron in the nucleus of the hydrogen atom doubles the atomic mass and decreases the intermolecular vibration. This phenomenon leads to increase in hydrogen bond strength (Chen et al., 1997). The protection of protein and DNA molecule may play a major role in dry heat resistance. However, the mechanism of increased survival of spores prepared by D_2O adaptation culture in contrast to H_2O culture at -20 °C is still unknown.

Spontaneous mutation rate of spores during storage

Significant increase in the spontaneous mutation rate was observed between D_2O and H_2O media after adaptation to 98% D_2O culture. No growth inhibition was shown by the adaptive D_2O culture process, so the possibility of well-known erroneous replication mechanism can be excluded immediately. D_2O has been reported to induce osmotic shock and to cause an efflux of intracellular potassium from algal cells (Andjus and Vucelic, 1990). Although, the physiological role of rapid K^+ excretion in response to different stresses is not yet fully understood, there is evidence that the redox state of cells may control the K^+ efflux through the membrane (Meury and Robin, 1990). A previous experiment has shown the cross-adaptation of bacterial cells to D_2O, oxidative and osmotic stresses (Nevo et al., 2004). The decreased level of intracellular potassium in bacterial cells exposed to oxidative stress may cause DNA relaxation and activate the DNA repair systems (Tkachenko et al., 1999), resulting in the declining spontaneous mutation rate during adaptation to 98% D_2O medium when compared with H_2O medium. Some studies in genetics and evolutionary biology have recently found a decreased mutation rate during latency when compared with the active disease or in a logarithmically growing culture over the same period of time. The pattern of polymorphisms suggests that the mutational burden *in vivo* is due to the oxidative DNA damage (Ford et al., 2011). Although, the decreased spontaneous mutation of spores by D_2O culture can be attributed to the heavy isotope resistance to reactive oxygen species, the mechanism of the dramatically reduced spontaneous mutation rates of spores by D_2O medium when compared with the H_2O medium during storage is still unknown. Spores were preserved by vacuum freeze drying and were sealed in a sand tube, so the reactive oxygen damage to DNA molecular was averted. This finding shows that D_2O has a protective effect on spore against damage to DNA molecular. Forty years ago, Löwdin (1963) proposed a quantum chemical mechanism for DNA mutation, which was based on inter-base double proton transfers (DPT). Löwdin argued that the inter-base protons are not classical particles but “wave packets” which obey the rules of quantum mechanics. Due to the quantum-mechanical “tunneling effect,” there is always a small finite probability that the protons will change places, which will alter the genetic code and give rise to mutation. According to the quantum theory of tunneling effect, the DPT rate does not only depend on the height and form of the proton transfer barrier but also on the mass of the proton and the environmental temperature as well. If the proton is replaced by deuterium, the DPT will slow down, which will reduce the DNA mutation rate. In addition, the high environmental temperature will increase the DPT rate, thus enhancing the DNA mutation probability. The Löwdin mutation mechanism was primarily supported by quantum chemical calculations and chemical experimental observations, proving that DPT indeed exists in base pairs (Florián et al., 1996; Lin et al., 2011; Cerón-Carrasco et al., 2009). Recently, this mechanism has been further supported by genomic analysis (Lind et al., 2008; Lynch et al., 2008; Ossowski et al., 2010; Fu et al., 2011). The analyses on the numerous genomes revealed the existence of a universal G: C→ A: T mutation bias in archaea, bacteria, fungi, plants and animals which cannot be sufficiently explained by any well-known DNA mutation mechanism. However, according to the Löwdin mutation mechanism, DPT occurs more frequently within GC pairs than within AT pairs because at the former, DPT has more favorable energy. Thus, the Löwdin mutation mechanism provides a ready explanation to the universal G:C→A:T mutation bias in the three superkingdoms of life, which clearly shows the special value of the Löwdin mutation mechanism. This model is not in conflict with any other DNA mutation model and is very appropriate to serve as a basic framework to establish a more accurate theory for the mechanisms of mutation.

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