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Original Article

Effects of Deuterium Oxide on *Streptococcus mutans* and *Pseudomonas aeruginosa*

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Abstract

A complex aggregation of microorganisms growing on a solid substrate is termed a biofilm and is considered to be an etiological agents. *Pseudomonas aeruginosa* and *Streptococcus mutans* are representative bacteria in such biofilms. It is well known that deuterium oxide (D_2O) causes toxic effects on a number of biological systems. We investigated the effects of D_2O on growth and biofilm formation of *P. aeruginosa* and *S. mutans*. These bacteria were incubated in medium containing D_2O (100%, 75% or 0%) at 37°C for 24 hr, 48 hr or 72 hr. Growth of *P. aeruginosa* was inhibited by D_2O within the first 48 hr. However, after 72 hr, growth rate was seen to increase in the D_2O -containing medium compared with in medium without D_2O . In contrast, the growth of *S. mutans* in the D_2O medium was inhibited within 72 hr. The biofilm formation of *P. aeruginosa* was increased in the D_2O medium. Biofilm formation of *S. mutans* in the D_2O medium increased compared with in the medium without D_2O , but this increase was only temporary in the case of *P. aeruginosa*. Compared to biofilm formation in 0% D_2O medium marked as 100%, the biofilm formation rate of *S. mutans* in 75% D_2O medium was 143% at 24 hr, 146% at 48 hr and 130% at 72 hr. In other D_2O concentration media biofilm formation was lower. In 100% D_2O medium, biofilm formation rate decreased from 114% at 24 hr to 56% at 72 hr. The biofilm formation rate of *P. aeruginosa* in 100% D_2O medium was 172% at 24 hr, but decreased to 88% at 72 hr. Biofilm formation of *P. aeruginosa* in 75% and 0% D_2O media showed no significant difference. We consider that these results were due to stress or alteration in bacterial metabolisms.

Key words: Deuterium oxide—*Streptococcus mutans*—*Pseudomonas aeruginosa*—Biofilm

Introduction

Accumulated data show two major effects of deuterium oxide (D_2O) on the living

system: the “solvent isotope effect (SIE)” and the “deuterium isotope effect (DIE)”¹⁶⁾. The SIE is due to the properties of D_2O itself, *i.e.*, its high density ($\rho=1.11$) compared to

H₂O ($p=0.99$) at 20°C and its high viscosity ($p=1.25$) compared to H₂O ($p=1.01$) at 20°C⁹⁾. The SIE involves the structure of water and macromolecules. On the other hand, the DIE involves the ability of D₂O to replace H with D in biological molecules: the stretching frequency of the O-D bond is approximately half that of the O-H bond, thus making C-D, O-D, N-D and S-D bonds stronger than their protonated forms (C-H, O-H, etc.)^{8,32)}. Although it is not easy to distinguish between the SIE and DIE, long-term effects are probably due to the DIE and short-term effects to the SIE. The DIE involves the metabolic reaction through cleavage of C-H bonds, and if growth and cell division take place in the presence of D₂O, some deuterated molecules will form during over periods of days, weeks, or longer. In contrast, the SIE involves isolated cells or enzymes over a much shorter period. The toxic effects of D₂O on the nervous system, liver and formation of different blood cells have been studied since the 1960s²⁹⁾. At the cellular level, D₂O affects mitosis and membrane function. Mice and rats which were forced to drink D₂O instead of H₂O died 7 days later^{12,13)} and higher concentrations (ca. 90%) of D₂O rapidly killed fish, tadpoles and drosophila²⁰⁾.

On the other hand, microorganisms are more tolerant to D₂O than multicellular systems³⁰⁾. Algae and bacteria can grow in 100% D₂O and utilize a large number of deuterated molecules¹⁴⁾.

Recently, bacterial biofilms have been considered a form of microbial ecology. Biofilms are packed communities of microbial cells which grow on surfaces and surround themselves with secreted polymers. Biofilms allow microorganisms to survive in hostile conditions, thus their character is significantly different from their planktonic counterparts. Differences are noticed mainly in their growth rate, biochemical composition, virulence and increased resistance to chemical antimicrobials^{2,5,17,24)}. Bacterial biofilms are well-organized structured communities. Some investigators describe biofilm as a thin basal layer on the substratum, with columnar, mushroom-shaped

multibacterial extensions into the lumen of the solution, separated by regions called channels seemingly empty or filled with extracellular polysaccharide^{3,28)}. These channels are considered to be at work as a selective transporter of low molecular materials such as water. Biofilm formation is related to the virulence of bacteria²⁴⁾.

Streptococcus mutans is a facultatively anaerobic Gram-positive bacterium known to be a cariogenic, and possesses many etiological agents, including the ability to form biofilm. Fermentation is the only metabolic reaction required for this bacterium to manufacture ATP. The matrix of biofilm by *S. mutans* is considered to be alpha-1-3-glucan. *Pseudomonas aeruginosa* is well known as the pathogen of hospital infection. *P. aeruginosa* is respiratory gram negative bacteria. The matrix of biofilm formed by *P. aeruginosa* is considered to be alginate. These two bacteria form different types of biofilm, and their respective pathogenicities are linked to that ability.

In this study, we investigated the effects of pure D₂O on the growth and biofilm formation of two types of biofilm-producing bacteria, *S. mutans* and *P. aeruginosa*.

Materials and Methods

1. Bacterial strain

S. mutans Ingbritt and *P. aeruginosa* GTC2 strains were used in this study. Bacteria were grown on Trypticase soy agar (Becton, Dickinson and Company, Maryland, USA) at 37°C in an aerobic atmosphere for 48 hr.

2. Media and growth conditions

As a base medium Trypticase soy broth (Becton, Dickinson and Company) was dissolved in each solvent at a concentration of D₂O 100%, 75% or 0%. Density was adjusted to 3%, and the medium supplemented with 5% sucrose for *S. mutans* and 1% glucose for *P. aeruginosa*. Under the same conditions, basal medium without glucose or sucrose was also used. These media were sterilized by filtration. Bacterial colonies were picked up from

Trypticase soy agar (Becton, Dickinson and Company) and suspended in each broth. The suspension was adjusted to OD 0.02 at 655 nm. One hundred μl suspension was then dispensed in 96-well polystyrene plates. Three plates of each type of bacteria were prepared and the plates incubated aerobically at 37°C for 24hr, 48hr or 72hr.

3. Bacterial growth

Bacteria floating in the medium or adherent to the plastic wells were all considered growing bacteria. Optical density, which is related to bacterial growth, was measured at 655 nm using the Microplate reader (Model 680; Bio-Rad Laboratories, Richmond, CA, USA). The number of viable bacteria was determined by resazurin reduction using Alamar blue (AbD Serotec, Oxford, UK). One hundred μl Almar blue, was added to each well and incubated at 37°C for 30 min. The optical density of the samples was measured at 540 nm using the Microplate reader³¹.

4. Semiquantification of biofilm

Bacteria adherent to the plastic wells were considered to be forming a biofilm⁴. The semiquantification of biofilm formation was carried out using toluidine blue by the method of Tanaka with some modifications²⁷. After measuring bacterial growth, the supernatant was decanted and wells washed twice with distilled water. Each well of the tissue culture plate was incubated at room temperature for 30 min with 100 μl of 0.1% toluidine blue to stain the biofilm. The excess stain was removed by washing twice with distilled water. One hundred μl ethanol was added to the well and left for 30 min to extract the stain. The optical density of samples was measured at 595 nm with the Microplate reader.

5. Statistical analysis

The relationship between growth and biofilm formation rate at each D₂O concentration for 24hr, 48hr or 72hr was analyzed using a one-factor ANOVA. Pairwise comparisons among the three concentrations of D₂O were made using the Tukey-Kramer test.

Results

1. Bacterial growth

Figures 1 and 2 show the results of optical density. Figures 3 and 4 show the viable number of bacteria.

Figures 1 and 3 show the progress of *P. aeruginosa* growth with time. Delayed growth was observed in both basal media and glucose media including D₂O. *P. aeruginosa* was pigmented green in 72hr (data not shown). *P. aeruginosa* was pigmented particularly strongly in the glucose-supplemented medium. This pigmentation was considered to influence optical density. Optical density of the media with glucose decreased at 72hr (Fig. 1C). This may have been due to the influence of pigmentation. While the number of viable *P. aeruginosa* in the H₂O medium with glucose showed peak growth at 24hr, the number of viable *P. aeruginosa* in the D₂O media with glucose showed peak growth at 48hr (Fig. 3C). At 48hr, D₂O medium showed a higher rate of activity of resazurin reduction than H₂O medium.

Figures 2 and 4 show *S. mutans* growth. The growth of *S. mutans* in D₂O media was not delayed, but always lower than that in H₂O. The number of viable cells was quite different in both the D₂O and H₂O media when supplemented with sucrose (Fig. 4C). In the basal medium, rates of activity of resazurin reduction of *S. mutans* showed no significant difference between various concentrations of D₂O at 72hr (Fig. 4B).

Figures 1B, D, 2B and D show the bacterial growth rates for each experimental condition compared to the growth in 0% D₂O medium (marked as 100%). Within the first 48hr, the growth of *P. aeruginosa* was inhibited by D₂O. However, after 72hr, the growth rates reached almost 130% in 100% D₂O medium and 110% in 75% D₂O medium (Fig. 1D). Growth of *P. aeruginosa* in 0% D₂O medium showed a decline. The growth of *S. mutans* in 100% and 75% D₂O medium was inhibited by D₂O within 72hr (Figs. 2C, D).

2. Biofilm formation

Figures 5A and C show amounts of biofilm

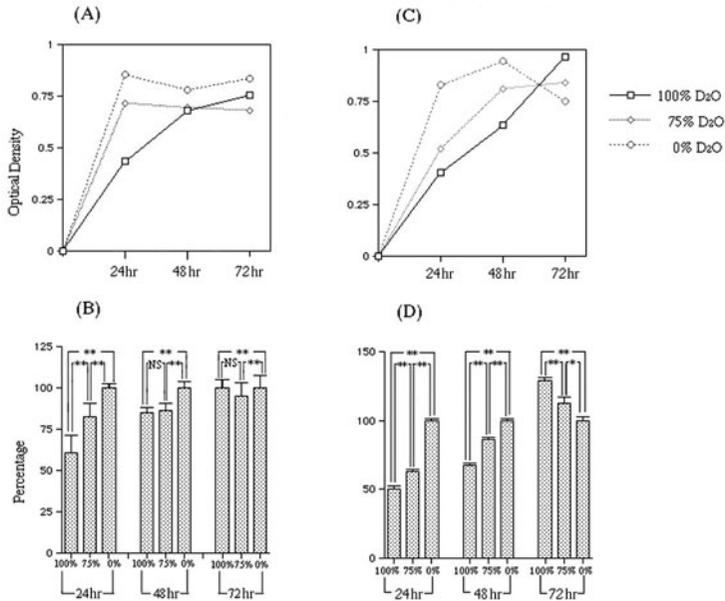


Fig. 1 (A) Medium turbidity of *P. aeruginosa* in basal medium without glucose. (B) Ratio of medium turbidity of *P. aeruginosa* in basal medium without glucose. (C) Medium turbidity of *P. aeruginosa* in basal medium with glucose. (D) Ratio of medium turbidity of *P. aeruginosa* in basal medium with glucose. Bar indicates standard error obtained from three separate assays. **: $p < 0.01$, *: $p < 0.05$ (Tukey-Kramer).

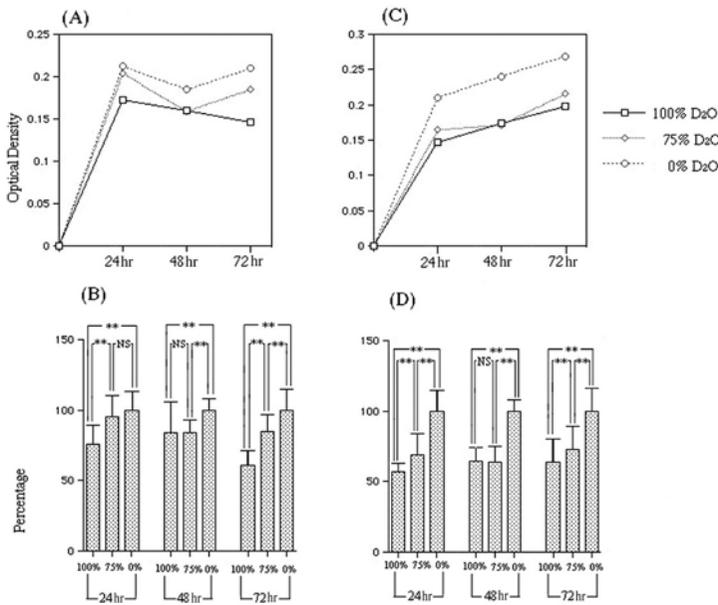


Fig. 2 (A) Medium turbidity of *S. mutans* in basal medium without sucrose. (B) Ratio of medium turbidity of *S. mutans* in basal medium without sucrose. (C) Medium turbidity of *S. mutans* in basal medium with sucrose. (D) Ratio of medium turbidity of *S. mutans* in basal medium with sucrose. Bar indicates standard error obtained from three separate assays. **: $p < 0.01$.

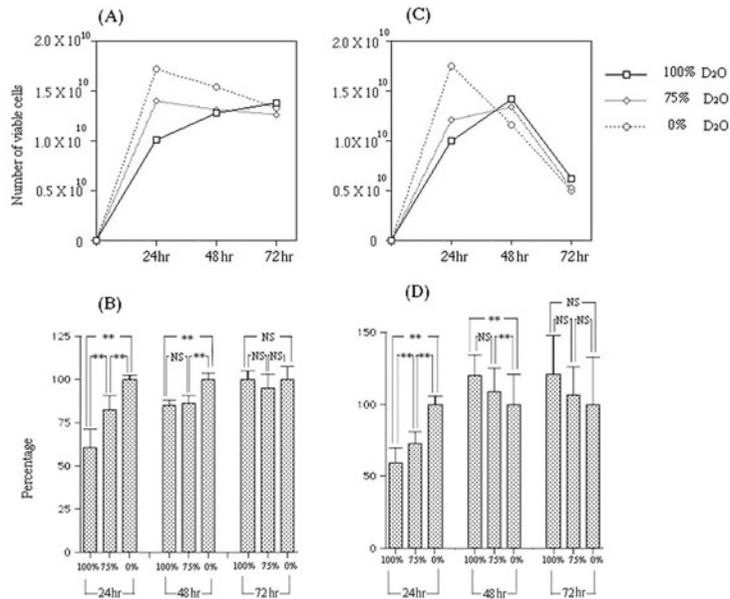


Fig. 3 (A) Number of viable cells of *P. aeruginosa* in basal medium without glucose. (B) Ratio of activity of resazurin reduction of *P. aeruginosa* in basal medium without glucose. (C) Number of viable cells of *P. aeruginosa* in basal medium with glucose. (D) Ratio of activity of resazurin reduction of *P. aeruginosa* in basal medium with glucose. Bar indicates standard error obtained from three separate assays. **: p < 0.01 (Tukey-Kramer).

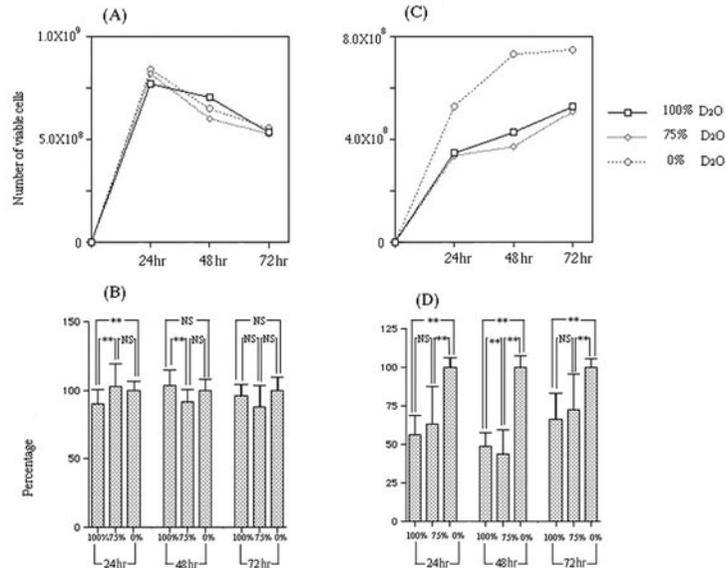


Fig. 4 (A) Number of viable cells of *S. mutans* in basal medium without sucrose. (B) Ratio of activity of resazurin reduction of *S. mutans* in basal medium without sucrose. (C) Number of viable cells *S. mutans* in basal medium with sucrose. (D) Ratio of activity of resazurin reduction of *S. mutans* in basal medium with sucrose. Bar indicates standard error obtained from three separate assays. **: p < 0.01 (Tukey-Kramer).

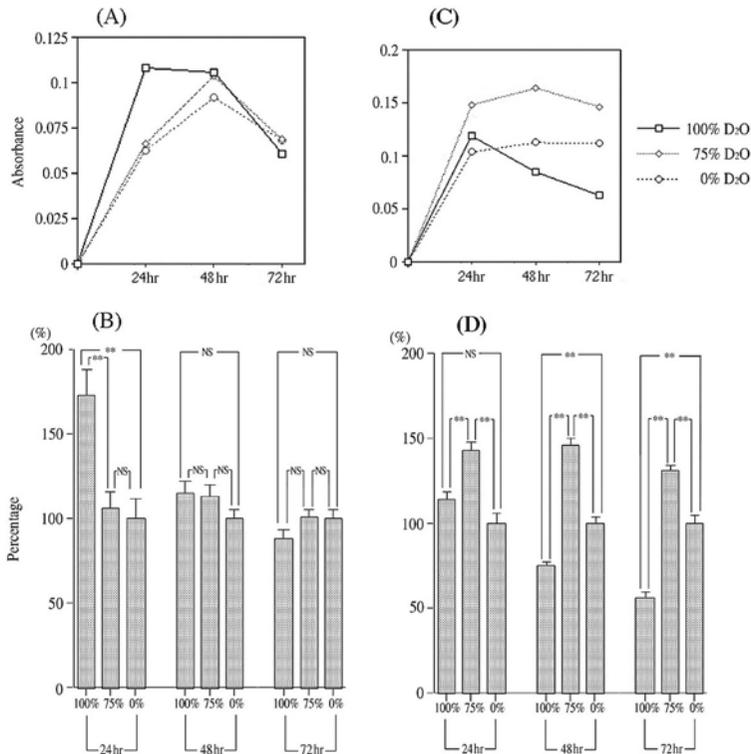


Fig. 5 (A) Amount of biofilm formation of *P. aeruginosa* in basal medium with glucose. (B) Biofilm formation rates of *P. aeruginosa* in basal medium with glucose. (C) Amount of biofilm formation of *S. mutans* in basal medium with sucrose. (D) Biofilm formation rates of *S. mutans* in basal medium with sucrose. Bar indicates standard error obtained from three separate assays. **: $p < 0.01$ (Tukey-Kramer).

measured by staining. *P. aeruginosa* formed much larger amounts of biofilm in the 100% D₂O medium than in the H₂O media at 24 hr, although this evened out by 48 hr. By 72 hr, the amount of biofilm in both media began to decline at the same rate. A comparison with *S. mutans* in Figs. 5A and C revealed a similar pattern in terms of decline in amount of biofilm formation. Figure 5B, however, revealed no remarkable increase in amount of biofilm with culture in 100% D₂O medium.

Figures 5B and D show biofilm formation rates compared to that in 0% D₂O medium (marked as 100% biofilm formation). The biofilm formation rate of *P. aeruginosa* in 100% D₂O medium was 172% at 24 hr, but

decreased to 88% at 72 hr. Biofilm formation in 75% and 0% D₂O medium was not significantly different (Fig. 5B). The biofilm formation rate of *S. mutans* was higher in 75% D₂O medium than in other D₂O concentration media: 143% at 24 hr, 146% at 48 hr and 130% at 72 hr. In 100% D₂O medium, biofilm formation rate decreased from 114% at 24 hr to 56% at 72 hr (Fig. 5D).

In 100% D₂O medium, the biofilm formation of both bacteria decreased gradually.

Discussion

Deuterium oxide has been considered harmless to bacteria because no influence

on bacterial growth was observed without any extended lag phase¹⁴. Recent reports suggest that D₂O affects bacterial metabolism^{11,19}. *Escherichia coli* revealed D₂O at log-phase, with 53% of intracellular water being replaced by D₂O¹⁵. Intracellular D₂O affects the increase of the anaplerotic supply of tricarboxylic acid cycle¹¹.

In *Rhodobacter sphaeroides*, the cytochrome C oxidase catalytic cycle was slowed down by deuterium isotope addition¹⁹. Cytochrome C oxidase is the terminal enzyme of the respiratory chain, and various aerobic bacteria, including *P. aeruginosa*, also possess this enzyme²².

In this study, D₂O (100%, 75%) inhibited growth of *P. aeruginosa* by approximately 50% compared to 0% D₂O for the first 24hr. However, the growth rates in 100% and 75% D₂O gradually recovered at 48hr, finally exceeding growth in 0% D₂O. These results possibly indicate that D₂O does not absolutely inhibit the respiration of *P. aeruginosa*. However, inhibition was limited until 48hr. At 72hr, media containing D₂O did not inhibit, but rather facilitated the bacterial growth. This may be due to the change in respiration metabolism of *P. aeruginosa*. The growth of *S. mutans* was inhibited during all test periods by D₂O (Figs. 2C, D). The growth pattern differed from that of *P. aeruginosa*. This may be related to the lack of the TCA cycle and respiration in *S. mutans*. It seems that D₂O does not completely terminate bacterial respiration.

Biofilm formation increases under some types of stress. Biofilm formation by microorganisms of the genus *Pseudomonas* is associated with various stress factors such as oxidative stress¹⁸, high temperature²⁵, hydrodynamic stress²³ and small amounts of ethanol or antibiotic^{6,7,10}. The biofilm formation of *S. mutans* also increases by various stress factors such as oxidative stress, nutritional stress and acid circumstance²¹. In many cases, stress from the environment induces bacteria to form much larger amounts of biofilm. Biofilm may play a role in defending bacteria against stress. In this study, D₂O inhibited bacterial growth for a short term, suggesting

that it acted as a stressor on the bacteria, thus inducing biofilm formation.

Biofilm formation is also led by the quorum sensing (QS) system. The QS system depends on bacterial cell density. When the population reaches a critical threshold density, bacteria communicate with one another and secrete specific signaling molecules. The signaling molecules in QS systems are, in many cases, acylated homoserine lactones (AHL) in Gram-negative bacteria, small peptides in Gram-positive bacteria or autoinducer-2 (AI-2), which is used both in Gram-negative and Gram-positive bacteria as a universal bacterial language³³. Deuterium isotope affects the metabolism of serine¹. However, it is not clear how the production of homoserine lactone is related to the metabolism of serine altered by D₂O. The QS signal of *S. mutans* is a 21-amino acid peptide phenomenon, a competent stimulating peptide^{21,34}. Although this peptide includes 4 serines, it is quite different from homoserine lactone, and the sensor of histidine kinase is associated with this peptide^{26,34}. It seems that D₂O is not involved in the metabolism of competent stimulating peptides.

Both of the two hypotheses regarding various types of stress and QS proposed above seem to indicate that the both are able to explain our results in this study. They both indicate that the D₂O effects on the bacteria observed in the present study was due to SIE, as SIE changed little and in a dose-dependent manner. Since we do not yet have clear evidence, further study is needed to clarify the stress effects of D₂O on bacteria.

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