ORIGINAL

Sterilization effect of UV light on *Bacillus* spores using TiO2 films depends on wavelength

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Abstract : UV light and photocatalysts such as titanium dioxide (TiO_2) and silver (Ag) are useful for disinfection of water and surfaces. However, the effect of UV wavelength on photocatalytic disinfection of spores is not well understood. Inactivation of *Bacillus* spores has been examined using different UV wavelengths and TiO_2 or TiO_2/Ag composite materials. The level of UVA disinfection of *Bacillus anthracis* and *Bacillus brevis* vegetative cells increased with the presence of the TiO_2 and Ag photocatalysts, but had little effect on their spores. *B. brevis* spores were slightly more sensitive to UVB and UVC than the spores of *B. atrophaeus*. Photocatalytic sterilization against spores was strongest in UVC and UVB and weakest in UVA. The rate of inactivation of *Bacillus* spores was significantly increased by the presence of TiO_2, but was not markedly different from that induced by the presence of Ag. Therefore, TiO_2/Ag plus UVA can be used for the sterilization of vegetative cells, while TiO_2 and UVC are effective against spores. J. Med. Invest. 59 : 53-58, February, 2012

Keywords **:** *disinfection, Bacillus spore, UV light, photocatalytic.*

INTRODUCTION

Effective disinfection procedures are central to the safety of public water systems, not only for drinking and sanitation, but also industrially, as biofouling is a commonplace and serious problem. Some bacteria (*e.g.*, *Bacillus* and *Clostridium* species) have vegetative and spore forms in the life cycle (1, 2). Thus, killing both the vegetative cells and spores is important for disinfection. When the environment becomes more favorable, spores can reactivate to form vegetative cells (3). Spores are dormant, tough, and temporarily non-reproductive structures that can survive without nutrients. They are commonly found in soil and water, where they may survive for long periods of time, and are resistant to ultraviolet radiation, desiccation, high temperature, extreme freezing, and chemical disinfectants (4-6).

Bacillus species are gram positive, aerobic, and spore forming bacteria (7). *Bacillus* spores are 5 to 50 times more resistant to UV radiation than the corresponding vegetative cells, and the mechanisms for

Received for publication August 31, 2011 ; accepted September 15, 2011.

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handling UV damage are different between the two life forms (8). *Bacillus anthracis* has nearly worldwide distribution, existing in the soil in the form of extremely stable spores and causing infection in farm and wild animals that have grazed on contaminated land or ingested contaminated feed (9). *Bacillus atrophaeus* is used as a biological indicator to monitor sterilization processes and as a surrogate in the development of biosafety methods (9-11).

Advanced oxidation processes using photocatalysts are potential alternatives to traditional methods of disinfection (12). TiO_2 or TiO_2 plus Ag are widely used as a photocatalyst because it is highly efficient, nontoxic, chemically and biologically inert and photostable, inexpensive and has good mechanical hardness (12, 13). However, the effect of TiO_2 and TiO_2 with Ag photocatalysts on bacterial spores is not well understood.

Many studies have shown that activation of TiO_2 and TiO_2 with Ag photocatalysts with ultraviolet A (UVA) light is a highly effective process for complete inactivation of bacterial vegetative cells of Gram-negative (*Escherichia coli*) and Gram-positive (*Staphylococcus aureus, Bacillus atrophaeus*) bacteria (14-17). The photocatalytic effect is reportedly dependent on the wavelength of light (18). UV is separated into UVA, UVB, and UVC based on wavelength. A high dose of UVA, which is used in the UVA-LED disinfection system, can kill vegetative cells directly (19). However, it is not clear that photocatalysts plus UVB or UVC exposure affect the disinfection of spores.

In this study, vegetable cells and spores of *B. atrophaeus* and *B. brevis* were used as models to investigate the sterilization capacities of TiO_2 and Ag with different UV wavelengths.

MATERIALS AND METHODS

Preparation of catalysts

The preparation of TiO_2 and TiO_2/Ag films has been described elsewhere (11). Briefly, TiO_2 and TiO_2/Ag films were prepared using the following chemicals : *tetra-isopropoxytitanium* $Ti(i\text{-}OC_3H_7)_4$ (Kantokagaku, Tokyo, Japan), *silver paste* (Ag) (Nippon Paint Corp., Osaka, Japan), *ethanol* (C_2H_5OH), *hydrochloric acid* (HCl), and H_2O. The TiO_2 sol was composed of 28.4g $Ti(i\text{-}OC_3H_7)_4$, 0.1 L C_2H_5OH, 2.32 g H_2O, and 0.514 g HCl. The solution was mixed for 5 min after the addition of 100 g Ag nanoparticle paste (Ag nanoparticle paste included 9 wt% protective colloid, 74 wt% C_2H_5OH, 17 wt% (0.1564 g) Ag nanoparticle) at room temperature (for TiO_2/Ag film). The diameter of nanoparticles in the paste was 20 nm. The glass substrates (76×26 mm, 1.2~1.5 mm thick) were immersed in sol solution at the rate of 2.25 mm/s and dried at room temperature for 10 min. The sol films were calcined at 250°C for 60 min in air to achieve cohesion and adhesion of the film to the substrate.

Preparation of spores and vegetative cells

Bacillus atrophaeus strain ATCC9372 and *Bacillus brevis* strain IFO3331 were used as model microorganisms for the disinfection experiments. The bacteria were cultured in Luria-Bertani (LB) broth (1% tryptone, 0.5% yeast extract, 1% NaCl) at 37°C with rotary shaking for 18 hr. The 2 ml overnight culture was centrifuged at 12,000 rpm for 3 min. The supernatant was discarded and the bacterial pellet was washed three times with sterilized phosphate-buffered saline (PBS) and suspended in PBS at an initial concentration of 10^8 CFU/ml.

Spores were prepared by growing *B. atrophaeus* and *B. brevis* on Luria-Bertani (LB) agar (1% tryptone, 0.5% yeast extract, 1% NaCl) plates at 37°C for at least 7 days (*B. atrophaeus*) and 15 days (*B. brevis*). Once spores had been collected by scraping the surface of the agar, they were washed three times with sterilized phosphate-buffered saline (PBS), suspended in PBS at an initial concentration of 10^8 CFU/ml. The spores were heat treated (80°C, 20 min) and stored in a refrigerator (4°C).

An aliquot (50 μl) of the bacterial spore or vegetative cell suspension was added drop-wise onto the surface of each film and exposed to UV light. The bacterial spore solution was not stirred during exposure time, which was performed in a dark room at 25°C for various time periods. After irradiation, the sample was placed in a petri dish with 5 ml of PBS and shaken for 10 min. Dilutions were placed on LB agar plates and incubated at 37°C for 24 hr before bacterial colonies were counted. Survival of the bacterial population was calculated using the equation :

Log survival ratio = $\text{Log}\ (N_t/N_0)$

Where N_0 represented the initial population and N_t represented the population after irradiation time (T). All results were calculated with data from three independent experiments.

UVA irradiation

Ultraviolet A-light emitting diodes (UVA-LED)

with peak irradiance at 365 nm (Model NCSU033B, Nichia Corp., Tokushima, Japan) were used to create the sterilization device. With one connection to the power source, the device ran on 20.00 V with a maximum current of 0.5 A and UVA intensity of 9 mW/cm^2, which was measured by the UIT-250 UV meter (Ushio Corp., Tokyo, Japan). In all cases, the light was switched on 30 min before the start of the reaction to stabilize the emission power and spectrum. The distance between the UVA-LED and the surface of the bacterial solution was 1.5 cm. UVA irradiation was performed in a dark room at 25°C for various time periods.

UVB and UVC irradiation

A low-pressure UV lamp (8 W ; 3UV Multi-Wavelength Lamp, 3UV-38 ; UVP, Inc. Upland, CA, USA) was used to irradiate at 302 nm (UVB) and 254 nm (UVC). Intensity was adjusted by changing the distance between the lamp and the bacterial spore solution. The intensity at 254 nm (distance : 46.5 cm) was 0.06 mW/cm^2, which was measured by the UIT-250 UV meter (Ushio Corp., Tokyo, Japan). Intensity at 302 nm (distance : 36 cm) was 0.09 mW/cm^2, which was measured by the MCPD-3700 multi channel photodetector (Otsuka electronics Co., Ltd, Osaka, Japan). In all cases, the light was switched on 30 min before the start of the reaction to stabilize the emission power and spectrum. UVB and UVC irradiation was performed in a dark room at 25°C for various time periods.

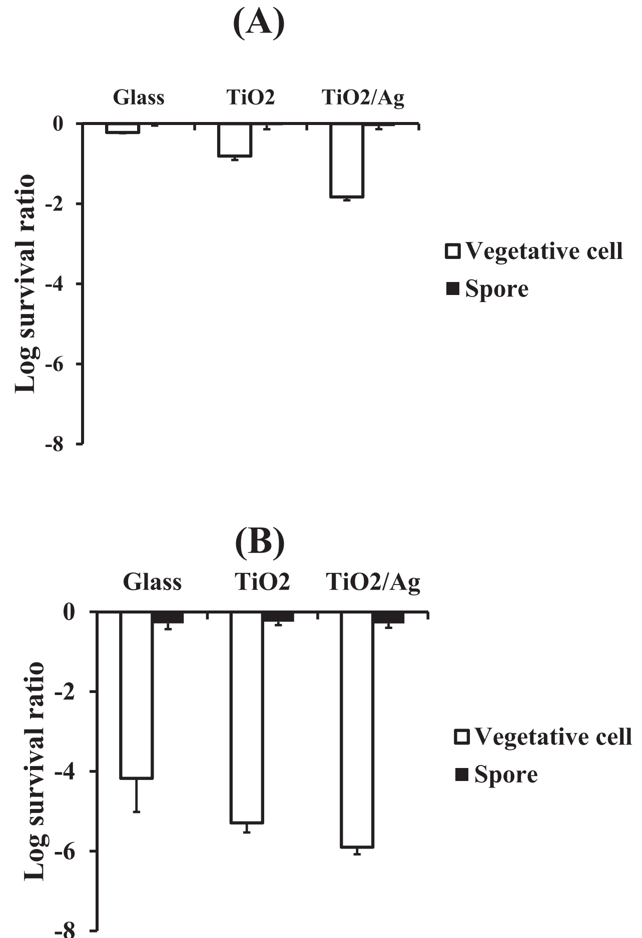

Fig. 1. Effect of photocatalysis on the inactivation of *B. atrophaeus* (A) and *B. brevis* (B). Bacterial spores and vegetative cells were exposed to UVA-LED at 365 nm. The irradiation dose was adjusted to 9 mW/cm^2, with an exposure time of 10 min. All experiments were performed in the dark at 25°C for the same period of time. Data are expressed as the means ± SD from three independent experiments.

RESULTS

The effect of the use of a photocatalyst and UVA on the inactivation of *B. atrophaeus* and *B. brevis* is shown in Figure 1. Inactivation was compared between the two forms of the bacteria. For vegetative cells, photocatalytic (TiO_2 or TiO_2/Ag films) inactivation of *Bacillus* were 4 to 9 fold (*B. atrophaeus*) and 1.2 to 1.4 fold (*B. brevis*) higher than when UVA exposure alone (normal glass) was used, which was consistent with previous observations (14-17, 20). In contrast, spores were resistant to UVA only exposure and photocatalytic inactivation. These results demonstrated that photocatalysis-assisted inactivation of vegetative cells is higher than inactivation of spores in the same organism when UVA is used. Moreover, *B. brevis* vegetative cells had a higher sensitivity to UVA exposure or photocatalytic inactivation than *B. atrophaus* in vegetative cells.

Next, we investigated the effects of UV wavelength and irradiation time on inactivation of *B. atrophaeus* and *B. brevis* spores (Fig. 2A, 2B). Bacterial spores were exposed to UVA-LED at 365 nm, a UVB lamp at 302 nm and UVC lamp at 254 nm. Exposure of UVA alone had very little effect on spores. But the bactericidal efficiency of UVB or UVC was increased with irradiation time in *B. atrophaeus* and *B. brevis* spores. With UVC exposure, disinfection of bacillus spore was significantly increased by the presence of TiO_2. These results indicate that photocatalyst function was involved in the inactivation spore mechanism with UVC, but neither is involved in the effects of UVA irradiation (Fig 2B and Fig 2C). Moreover, the rate of inactivation of *Bacillus* spores was significantly increased by the presence of TiO_2, but was not markedly different from the inactivation level induced by TiO_2/Ag (Fig 2C).

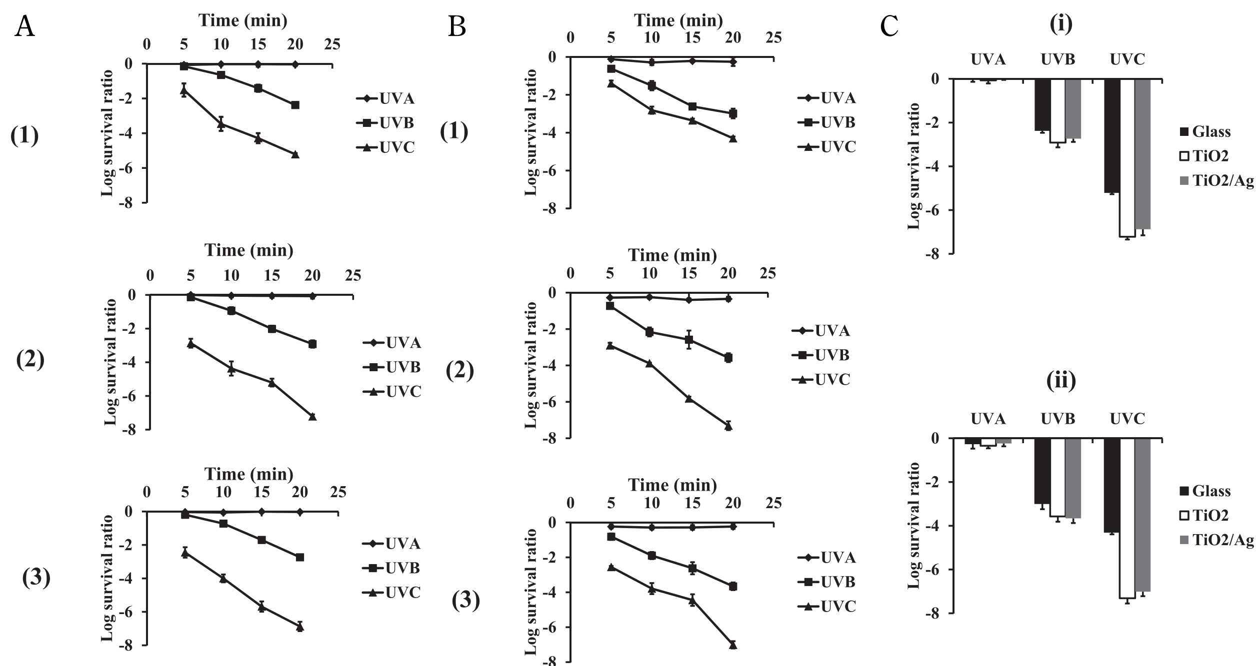

Fig. 2. Effect of wavelength and irradiation time on inactivation of *B. atrophaeus* (A) and *B. brevis* (B) spores. Comparison of the effect of photocatalysts (TiO_2, TiO_2/Ag) and a non-photocatalyst (normal glass) on the inactivation of *B. atrophaeus* (C(i)) and *B. Brevis* (C(ii)) spores. C(i) and C(ii) were re-made from same data of A and B for clear the differences of the effect of photocatalysts. Bacterial spores were exposed to UVA-LED at 365 nm, UVB lamp at 302 nm and UVC lamp at 254 nm. Doses were adjusted to 9 mW/cm^2 (UVA), 0.09 mW/cm^2 (UVB) and 0.06 mW/cm^2 (UVC). (I) Normal glass sheet, (II) TiO_2 film, (III) TiO_2/Ag film. All experiments were performed in the dark at 25°C for the same period of time. Data are expressed as means ± SD from three independent experiments.

DISCUSSION

While UVA disinfection of *B. atrophaeus* and *B. brevis* vegetative cells was increased by the presence of TiO_2 and Ag, inactivation of *B. atrophaeus* and *B. brevis* spores with TiO_2 and/or Ag was very low (Fig. 1). These results contradict reports showing an increase in the efficiency of photocatalytic inactivation of *Bacillus* spores with TiO_2 upon incorporation of silver with high doses of UVA 153 W/m^2 (21) and differences in the preparation of photocatalysts (21, 22). The spores of *Bacillus* species are much more resistant to UV radiation than are the corresponding growing cells, and mechanisms that respond to UV damage are different in the two populations (8). The UVA intensity in this study was 9 mW/cm^2, which was much higher than UVB (0.09 mW/cm^2) or UVC (0.06 mW/cm^2) doses. The high intensity of UVA irradiation needs higher energy consumption than UVB irradiation or UVC irradiation. Thus, we think that photocatalytic inactivation with UVA using thin films was hard to available to spore sterilization system. Under the conditions of this experiment, the spores of both species were more sensitive to UVB and UVC than UVA. UVC enhanced the rate of inactivation by approximately 2 fold over that of UVB. Previous studies have reported that UV fluency primarily affects radiation-sensitive microorganisms via DNA damage, whereas at higher UV fluency (various) mechanisms of protein damage are presumably responsible for inactivation (23).

UVA irradiation increased levels of reactive oxygen species, including superoxide anion radicals ($O_2^{\cdot-}$), hydroxyl radicals (OH·), hydrogen peroxide (H_2O_2), and singlet oxygen ($^1O_2$) (24-26). We reported UVA-induced oxidative damage is a major factor in the inactivation of bacteria, previously (19). The primary photocatalytic action for bacterial disinfection is also oxidative damage (27). The mechanism underlying photocatalysis, in the presence of TiO_2 and Ag, is irradiation of TiO_2 with UV light to produce electron-hole pairs. Absorption of photon by the electron in the valence band of TiO_2 promotes the electron to the conduction band, creating a negative-electron (e^-) and positive-hole (h^+) pair. Formation of the electron-hole pair leads to generation of reactive hydroxyl radicals (·OH) on the TiO_2 surface (28). Hydroxyl radicals are powerful oxidants that can damage microbial cell walls and

membranes, DNA and RNA (27). Moreover, the ability of TiO_2 to inactivate *Bacillus* spores was not markedly different in the presence of Ag. Although Ag itself has no killing activity (15), silver doping enhances electron-hole separation in TiO_2, the visible light excitation of TiO_2 and, by extension, the photocatalytic inactivation of microorganisms (29). It is speculated that Ag enhances the oxidative ability of TiO_2 photocatalytic action (30). Thus, we hypothesized that spores are more resistant to oxidative stress than vegetative cells (31). Because the oxidative resistance of spores is important in the sterilization process, further study is necessary.

In conclusion, the presence of photocatalysts (TiO_2 and Ag) increased the photocatalytic disinfection of *Bacillus* vegetative cells. The disinfection of spores depended on the wavelength of UV light. Photocatalysis had no effect on *Bacillus* spores when UVA light was used, but had a pronounced effect under UVC. In addition, the presence of TiO_2 enhanced the photocatalytic inactivation rate of spores.

CONFLICT OF INTEREST

None of the authors have any conflicts of interest to declare.

ACKNOWLEDGMENTS

This project was partly supported by JSPS Grants-in-Aid for Scientific Research/Scientific Research on Priority Areas, and JST A-STEP (Japan Science and Technology Agency Adaptable and Seamless Technology Transfer Program through target-driven R&D).

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