Role of Visible Light-Activated Photocatalyst on the Reduction of Anthrax Spore-Induced Mortality in Mice

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Abstract

Background: Photocatalysis of titanium dioxide (TiO₂) substrates is primarily induced by ultraviolet light irradiation. Anion-doped TiO₂ substrates were shown to exhibit photocatalytic activities under visible-light illumination, relative environmentally-friendly materials. Their anti-spore activity against Bacillus anthracis, however, remains to be investigated. We evaluated these visible-light activated photocatalysts on the reduction of anthrax spore-induced pathogenesis.

Methodology/Principal Findings: Standard plating method was used to determine the inactivation of anthrax spore by visible light-induced photocatalysis. Mouse models were further employed to investigate the suppressive effects of the photocatalysis on anthrax toxin- and spore-mediated mortality. We found that anti-spore activities of visible light illuminated nitrogen- or carbon-doped titania thin films significantly reduced viability of anthrax spores. Even though the spore-killing efficiency is only approximately 25%, our data indicate that spores from photocatalyzed groups but not untreated groups have a less survival rate after macrophage clearance. In addition, the photocatalysis could directly inactivate lethal toxin, the major virulence factor of B. anthracis. In agreement with these results, we found that the photocatalyzed spores have tenfold less potency to induce mortality in mice. These data suggest that the photocatalysis might injure the spores through inactivating spore components.

Conclusion/Significance: Photocatalysis induced injuries of the spores might be more important than direct killing of spores to reduce pathogenicity in the host.

Introduction

Naturally occurring anthrax is a disease acquired following contact with anthrax-infected animals or anthrax-contaminated animal products. The disease most commonly occurs in herbivores, which are infected by ingesting spores from the soil. For centuries, anthrax has caused disease in animals and, uncommonly, serious illness in humans throughout the world [1]. Research on anthrax as a biological weapon began more than 80 years ago [2]. Recently, the anthrax-letter attacks further evidenced this emerging terrorist threat, leading to renewed attention to the importance of prophylaxis, prevention and handling for anthrax [3]. Treatments or agents commonly cited to inactivate anthrax spores include heat, formaldehyde, hypochlorite solutions, chlorine dioxide, and radiation [4]. However, most of these treatments and reagents are hazardous to humans that limit their usage in public environments only after detecting the contamination sources, rather than prevention. Thus, a safer disinfection technique, which could exert a continuous antimicrobial effect in our living environment, would be highly desirable. Here, we present a visible light inducible photocatalyst which might provide a complementary and possibly alternative approach to meet this need.

Photocatalytic titanium dioxide (TiO₂) substrates have been shown to eliminate organic compounds and to function as disinfectants [5]. Upon ultraviolet (UV) light excitation, the photon energy excites valence electrons and generates pairs of electrons and holes (electron-vacancy in the valence band) that react with atmospheric water and oxygen to yield reactive oxygen species (ROS) such as hydroxyl radicals (·OH) and superoxide anions (O₂¬) [6]. Electron holes, ·OH and O₂¬ are extremely reactive and could react with cellular components and function as biocides [5]. Since pure TiO₂ photocatalyst is effective only upon irradiation by UV-light at levels that would induce serious damage to human cells, the potential applications of TiO₂ substrates for use in our living environments are greatly restricted. Recently, anion-containing anatase TiO₂ photocatalysts have been identified, which are activated by illumination with visible light [7,8],

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offering the potential to overcome this problem [9]. Previously, we demonstrated that nitrogen-doped TiO2 [TiO2 (N)] photocatalyst could significantly eliminate *Escherichia coli* and other mild pathogens [9]. However, its antimicrobial activity and the antimicrobial mechanism against biological weapons, such as *Bacillus anthracis*, has not yet been reported. In this study, the anti-spore activity of TiO2 (N) photocatalysts against *B. anthracis* was compared with several other bacillus species including *Bacillus subtilis*, *Bacillus thuringiensis*, and *Bacillus cereus*. Among these bacteria, *B. subtilis* is an insect pathogen [12]; and *B. cereus* causes broad clinical infections including local infections, septicemia, central nervous system infections, respiratory infections, endocarditis, pericarditis and food poisoning [13]. In this study, visible light-mediated photocatalysis was found to inactive 25%–40% spores of *B. subtilis*, *B. thuringiensis*, *B. cereus* and *B. anthracis*. Even though the efficiency of bacterial-killing is less than 1 log CFU, intriguingly, animal experiment revealed that the photocatalysis reduced more than ten times potency of *B. anthracis* spores to induce mortality in mice. To further investigate the underlining mechanism, here we analyzed photocatalyst-mediated inactivation of anthrax lethal toxin (LT) and the survival rate of spore after the macrophage clearance *in vitro*.

**Results**

UV-Vis absorption spectroscopic analysis

To investigate the physical properties of pure, carbon- and nitrogen-doped TiO2 films, respectively, X-ray diffraction (XRD) patterns and Raman spectra (data not shown) were obtained. Our results showed that all the films were anatase phase but the intensity of XRD and Raman peaks was slightly decreased in carbon and nitrogen doped films, indicating that the carbon/nitrogen incorporation induced decreasing crystallinity. The UV-Vis spectra of pure, carbon and nitrogen doped TiO2 films are shown in Fig. 1. The carbon and nitrogen substitution of oxygen in TiO2 caused the absorbance edge of TiO2 to shift to the higher wavelength region. The pure TiO2 film absorption edge which was at 380 nm gradually red-shifted to ~425 nm and ~565 nm in carbon and nitrogen doped TiO2 films, respectively. This shift in the visible region is the result of incorporation of carbon and nitrogen into the TiO2 network to form Ti-C and Ti-N bonds. Substitutional carbon or nitrogen atoms introduce new states (C2p or N2p) close to the valence band edge of TiO2 (i.e. O2p states). As a result of this the valence band edge shifts to higher energy compared with the reference TiO2 and the band gap narrows. The energy shift of the valence band depends on the overlap of carbon states and O2p states. A higher doping concentration of carbon or nitrogen results in higher energy shift due to significant overlap of carbon or nitrogen and oxygen states and this leads to a narrower band gap in the compound [14].

Bactericidal activities of TiO2 photocatalysts against *Bacillus subtilis*

Before the spore experiments, living bacteria were used to test the photocatalysis system. Since *B. anthracis* is a hazardous microorganism, we first used *B. subtilis* as a surrogate to determine the bactericidal activity of nitrogen-doped [TiO2 (N)] and carbon-doped TiO2 [TiO2 (C)]. We placed 1×104 CFU *B. subtilis* on different substrates including cover glass (silica, without TiO2 coating), and silica substrates coated with thin films of TiO2, TiO2 (N), and TiO2 (C). These preparations were then illuminated with visible light and the levels of surviving bacteria were quantified as previously described [9]. We found that TiO2 (N) exhibited a significantly better performance to reduce the number of surviving *B. subtilis* bacteria when compared to TiO2 and TiO2 (C) (Fig. 2, **P<0.01**).

To obtain dose dependent and kinetic data for *B. subtilis* on photocatalytic substrates, we further analyzed the effects of visible-light illumination at various distances (5 cm, 10 cm, 20 cm, and with respective illumination intensities of 3×104, 1.2×105, and 3×105 lux) or at various time points (Fig. 3). The results showed that TiO2 and TiO2 (C) substrates had no detectable bacterial-killing effect, while TiO2 (N) contained significantly greater bactericidal activity, by which it induced nearly a 1 log CFU reduction under 3×105 lux visible-light illumination for 25 minutes (Fig. 3A, 3B, *P<0.05*; **P<0.01**, compared to respective
TiO$_2$ groups). Although prolonged illuminations tended to increase the bactericidal effect of TiO$_2$ (C) substrates (25 min, Fig. 3B), the killing efficiency was still not statistically significant as compared with the TiO$_2$ groups.

**Anti-spore activities of TiO$_2$ (N) against Bacillus species**

Photocatalyst-mediated killing was performed to determine the bactericidal effect of photocatalysis on *B. cereus*, *B. thuringiensis* and *B. anthracis*. Compared to TiO$_2$ thin films, we found that TiO$_2$ (N) thin films were significantly more effective in killing the living *B. cereus*, *B. thuringiensis* and *B. anthracis* bacteria under visible light illumination (Fig. 4A, *P*<0.05, **P**<0.01).

In spore experiments, TiO$_2$ (N) also exhibited a better anti-spore activity than TiO$_2$ thin films, although this activity was less efficient (25–40% killing/inactivation) (Fig. 4B, *P*<0.05) compared to the results obtained in living bacteria experiments (Fig. 4A).

**Treatments of photocatalyzed spores and LT to mice**

A mouse model was further used to investigate whether these photocatalyzed spores are less pathogenic. Before the photocatalyst experiment, we determined anthrax spore mediated mortality in mice. We found that 50% mortality required a single inoculation of 1×10$^6$ CFU anthrax spores, and the mortality reached to 100% when 7.5×10$^6$ CFU spores were used (Fig. 5A).

In photocatalyst experiment, we found that visible-light induced photocatalysis on TiO$_2$ (N) but not pure TiO$_2$ thin-films significantly attenuated the ability of anthrax spores (1×10$^7$ CFU, before photocatalysis) to cause mortality in mice (Fig. 5B, *P*<0.05 and **P**<0.01 compared to respective TiO$_2$ groups).

The reduced pathogenicity of photocatalyzed spores could not be simply attributed to the reduction of viable spores. To explain this
phenomenon, we hypothesized that photocatalysis on TiO$_2$ (N) might not only eliminate 25% of the viable population but also injure the remaining spores through inactivating bacterial components. Such injuries could be resolved during germination of spores on cultural dishes but the repair process could not be accomplished in time to enable the bacteria escape from phagocytic clearance in mice. To investigate whether photocatalysis could inactivate protein components of $B$. anthracis, anthrax lethal toxin (LT), an example of bacterial proteins and the major virulence factor, was subjected to visible light activated photocatalysis on TiO$_2$ and TiO$_2$ (N) photocatalysts, respectively; spores in TiO$_2$ (N) groups induced less mortality in mice ($\Delta$) compared to untreated (○) or TiO$_2$ (▼) groups (B) (n = 6). Aliquots of anthrax LT (500 µg PA : LF = 5:1) was subjected to photocatalysis on TiO$_2$ and TiO$_2$ (N) photocatalysts, respectively; LT (100 µg/g) in TiO$_2$ (N) groups ($\triangle$) induced less mortality in mice compared to untreated (○) or TiO$_2$ (▼) groups (C) (n = 6).

In vitro phagocytic clearance analysis

Anthrax spore can multiply in phagocytes [17]. To investigate whether photocatalysis might injure the spores and make them vulnerable for the clearance by phagocytes and further handicap the bacterial amplification within phagocytes, photocatalyzed anthrax spores were then treated to macrophage J774A.1 cells. We found that spores in light illuminated-TiO$_2$ (N) groups did not increase the number of surviving bacteria within 24 hours (Fig. 7A, TiO$_2$ (N)+L 1 hr vs. 24 hr). By contrast, untreated spores, or spores from groups without light illumination, or spores from illuminated-TiO$_2$ groups were all significantly multiplied 3–4 fold within 24 hours (Fig. 7A, untreated/TiO$_2$-L/TiO$_2$ (N)-L/TiO$_2$+L, 1 hr vs. 24 hr, *P<0.05, **P<0.01). Since low level of surviving bacteria in the 24-hour groups might be also possible [15,16], did not decrease after photocatalysis (Fig. 6B). These data might suggest that protein degradation dose not play major role in the inactivation of LT.
attributed to the low phagocytic efficiency, to investigate whether photocatalysis might make these spores hard to be engulfed by phagocytes, we analyzed the remaining viable spores in the macrophage-culture medium. We found that the viable spores in the medium showed no significantly differences between each groups (Fig. 7B, 1 hr vs. 2 hr, 3 hr and 24 hr, all groups compared to each other), indicating that the low level of surviving bacteria in the TiO$_2$ (N)+L/24 hr groups is not attributed to the low phagocytic efficiency (Fig. 7A). These results suggest that visible-light induced photocatalysis on TiO$_2$ (N) substrates handicapped the amplification of anthrax in phagocytes.

**Discussion**

The antibacterial property of photocatalysts was primarily induced under UV irradiation [5,18,19], and more recently by visible-light illuminated conditions [9,20–23]. These studies provide valuable observations for the bactericidal activity of photocatalysts. Since these researches were mainly using laboratory *E. coli* strains as experimental materials, the potential application to apply on the eradication of spores of pathogenic bacteria, and especially the capability and the mechanism to reduce their pathogenicity were rarely discussed. Here we used spore forming bacillus bacteria as model systems to study the anti-spore activity of the visible-light photocatalyst. Since *B. anthracis* is a hazardous microorganism, in this study we first used *B. subtilis*, a bacillus bacterium with natural habitat in the soil that is not harmful to humans, as a surrogate for *B. anthracis*. We found that TiO$_2$ (N) exerted anti-spore effects against *B. subtilis* and all our tested Bacillus species including *B. cereus*, *B. thuringiensis* and *B. anthracis*, with similar killing-efficiencies among these bacteria. These results suggest that *B. subtilis*, *B. cereus*, *B. thuringiensis* might be useful as surrogates for further photocatalyst-mediated anti-arthrax research. Even though the spore-killing efficiency is not good enough as compared with other well-developed methods [4], the contrast between the low spore-killing and a relatively high reduction of pathogenicity, inspired us to further investigate the underlining mechanism, by which it led us to find that the photocatalysis could inactivate the LT and handicapped the spore to multiply in the phagocytes. This is a finding not yet been reported previously.

The molecular target of photocatalyst-induced damage on bacteria is rarely discussed. It is shown that bacterial membrane lipid components likely to be the cellular target of photocatalyst induced ROS [5]. Using living *E. coli* as a model system, the authors found that TiO$_2$-mediated photocatalysis promoted peroxidation of the polyunsaturated phospholipid component of bacterial membranes and then further led to respiratory activity loss and cell death [5]. Unlike vegetative cells, spores contain strong resistance to almost all antibacterial agents [4,26,27], as first observed by Koch over 100 years ago that *B. anthracis* spores could survive boiling. In the century that followed, it was learned that the protein components involving not only the composition of high density spore coat, a multilayered structure surrounding the spore that attributed to the resistance, but also the sensing responses to the renewed presence of nutrients in the environment, the condition under which the spore can convert to a growing cell through a process called germination [17,27,28]. Thus, the inactivation of a spore might be not just by disrupting the lipid components; deteriorated protein function could be involved as well. Photocatalysis-mediated protein dysfunction is rarely discussed. Evidence from enzyme-linked immunosorbent assays (ELISA) indicates that photocatalysis could affect the antigenic property of hepatitis B virus surface antigen and reduce its binding to specific antibody [29]. In this present study, we demonstrate the first time that photocatalysis could inactivate the bacterial exotoxin LT efficiently. Anthrax LT is a major virulence factor beneficial for the bacterium to establish initial infections in macrophages [30,31]. Our Western blotting analysis revealed that both PA and LF, two components of LT, remained intact after photoreactions, indicating protein degradation does not play a major role in the inactivation. Although evidences indicate that LT is not expressed in anthrax spores [32,33], since both protein toxin and spores are sensitive to photocatalysis, it seems likely that some of the spore proteins might be also sensitive to the photoreactions. Further investigation to identify the specific protein is needed.

Taken together, this study demonstrated that TiO$_2$ (N) substrates could inactivate both spores and toxin of *B. anthracis* under illumination by ordinary light sources such as incandescent lamps. Our results suggest that the suppressed amplification of *B. anthracis* in phagocytes might be more important than the direct killing for photocatalysts to reduce the pathogenicity of the spores. These concepts might provide a new prospect to develop next generation antimicrobial agents.

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**Figure 7. Surviving spores after clearance by macrophages.** Anthrax spores were treated to J774A.1 macrophage cells (MOI: 0.01 spores/cell). Surviving bacteria (CFU) that harvested from macrophage cell lysate (A) or macrophage cell cultural medium (B) were shown. Columns designated TiO$_2$ or TiO$_2$ (N) represent anthrax spores were pretreated with photocatalysis on TiO$_2$ and TiO$_2$ (N) substrates, respectively. Columns designated “+L” or “−L” represent experimental conditions with or without light illumination, respectively. *P*<0.05, **P*<0.01, compared to 1 hr groups of respective conditions (A).

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Materials and Methods

Preparation of TiO₂ substrates

Three types of films, TiO₂, TiO₂–Cₓ, and TiO₂–Nₓ, were prepared in an ion-assisted electron-beam evaporation system (Branchy Vacuum Technology Co., Ltd., Taoyuan, Taiwan). The distance between the rotating substrate holder and the electron-beam evaporation source was 550 mm. The chamber was evacuated with a mechanical pump (ALCATEL-2033SD, LACO Technologies, Salt Lake City, UT, USA) and a cryopump (Cryo-Torr® 8, ULVAC Cryogenics, Chigasaki City, Kanagawa Prefecture, Japan) to a base pressure below 2.7 × 10⁻⁴ Pa. The substrates used were polished Si (100), quartz and glass coupons, which were sputter-etched with argon ions (Ar⁺) for 5 minutes prior to the deposition to remove any residual surface pollutants. The substrate temperature was maintained at 300°C with a quartz lamp. The TiO₂ films were deposited in oxygen atmosphere (6.7 × 10⁻² Pa) using rutile TiO₂ (99.99%) as a source material. The nitrogen flow for TiO₂–Nₓ films was 15 standard cm³ min⁻¹ through the ion gun at a constant pumping speed and the chamber pressure was 4.4 × 10⁻² Pa. The carbon dioxide gas flow for TiO₂–Cₓ films was 7 standard cm³ min⁻¹ and the chamber pressure was 2.6 × 10⁻² Pa. The ion gun beam current of 10 mA and voltage of −1000 V was maintained by a Commonwealth Scientific IBS controller. Sufficient energy and current of the ion beam are critical to incorporate significant dopant concentration in the film. Without ion bombardment, it is difficult for the dopant to compete with the oxygen for incorporation into anatase titania. The deposition rate was adjusted to 0.2 nm s⁻¹ using a quartz crystal monitor for all films deposited at a thickness of 1.2 μm. The three types of films were prepared under the optimized conditions for their categories of anatase crystallinity and dopant concentration [34,35]. The structure and crystallinity of the films were investigated using a Rigaku D/MAX-2500V 18 kW low angle X-ray diffractometer (XRD) (Rigaku, Shibuya-Ku, Tokyo, Japan) operating with Cu-Kα radiation at 40 kV and 150 mA and a Renishaw 10000B Raman spectrometer equipped with a charge-coupled detector (CCD) and a CW 532 nm wave length diode pump solid state (DPSS) laser as the excitation source (Renishaw plc, Representative Office, Nantun District, Taichung, Taiwan). The UV-Vis absorption spectra were recorded on a Hitachi 3300H spectrophotometer (Hitachi Taiwan, Taipei, Taiwan).

Photocatalyzed Anthrax Spore

Bacterial strains and culture

B. anthracis (ATCC 14186), which contains both pXO1 and pXO2 plasmids that express functional lethal toxin (LT) and edema toxin (ET), was grown and maintained as previously described [16,36,37]. B. cereus (ATCC 13061) and B. thuringiensis (ATCC 35646) were maintained and cultured in nutrient agar or nutrient broth at 30°C [38,39], and B. subtilis (ATCC 39090) was maintained and cultured in trypticase soy agar or broth at 37°C [40]. All bacteria were stored in 50% medium and 50% glycerol solution in freezers at −80°C before use. To reactivate bacteria from frozen stocks, 25 μl bacterial stock solution was transferred to a test tube containing 5 ml of freshly prepared culture medium and then incubated at 30°C or 37°C under agitation overnight (16–18 hr). Spores of B. anthracis were prepared as previously described [41,42]. Overnight tryptic soy broth cultures of B. anthracis were diluted to about 10⁶ CFU/ml in phosphate-buffered saline, and 0.1-ml aliquots were inoculated onto blood agar plates. The agar plates were incubated at 25–37°C until 90–99% phase-bright spores were observed by phase-contrast light microscopy (see below). Spores were harvested and washed with cold sterile distilled ionized (DI) water as previously described [41] and stored in DI water at 4°C until use for up to 2 weeks, changing the water at least once a week, or in the freezer at −20°C for up to a month. The quality of spores was determined by two complementary criteria previously established to validate the presence of dormant spores [42]. The criteria consisted in the evaluation of (i) the absence of vegetative cells (rods) determined by microscopic examination as described, and (ii) the survival of spores in hydrochloric acid (2.5 N). Spore preparations of B. subtilis, B. cereus and B. thuringiensis were followed a similar protocol.

Photocatalytic reaction and detection of viable bacteria

In this study, bacterial concentrations were either determined by the standard plating method or inferred from optical density readings at 600 nm (OD₆₀₀). For each Bacillus species, a factor for converting the OD₆₀₀ values of the bacterial culture to concentration (CFU/ml) was calculated as follows. A fresh bacterial culture was diluted by factors of 10⁻¹ to 10⁻⁷, and OD₆₀₀ of these dilutions was measured. Bacterial concentrations of these dilutions were determined by the standard plating method. The OD₆₀₀ values were plotted against the bacterial concentration log values, and the conversion factors for the particular bacteria were calculated from three independent measurements. For example, the conversion factor for B. subtilis was calculated to be 1 × 10⁹ CFU/ml per OD₆₀₀ by this method.

In order to determine the bactericidal effects of the TiO₂-related substrates, 200 μl of bacterial overnight culture was transferred into 5 ml of culture medium and incubated at 37°C until an OD₆₀₀ of 0.3 to 0.6 (log phase) was reached. The bacterial concentrations were calculated using the previously determined conversion factor for the bacteria, and the cultures were diluted to 1 × 10⁵ CFU/ml with culture medium. One hundred microliters (1 × 10⁵ CFU) was then applied to an area of approximately 1 cm² of the different TiO₂-related substrates using a plastic yellow tip. The bacteria substrates were then placed under an incandescent lamp (Class I light, incandescent lamp, 60W, Philips, Taiwan) for photocatalytic reaction. A light meter (model LX-102, Lutron Electronic Enterprises, Taiwan) was used to record the illumination density. In the following photocatalysis experiments, the bacterial solution was supplied by 5 to 10 ml additional distill water every 5 minutes to maintain approximately 100 μl of total volume. After the photocatalyst-killing for 25 minutes, the bacteria containing solution approximately 85 μl was recovered from the photocatalyst substrates using a tip, additional 60 μl fresh medium was used to wash the remaining bacteria on the photocatalyst substrates. Two bacterial containing solutions were mixed, diluted and placed on agar plates. To test whether spores have different efficiency to adhere on TiO₂ substrates that might influence the photocatalysis result, we analyze the spore recovery rates from TiO₂, TiO₂ (C) and TiO₂, TiO₂ (N) substrates. We found that the recovery rates are similar (data not shown), which has no statistic significant among these groups. In the dose-dependence experiments, illumination was carried out for 5 min at distances of 5, 10, and 15 cm from the lamp, corresponding to the illumination intensities of 3 × 10⁴, 1.2 × 10⁴, and 3 × 10³ lux (lumen/m²)(90, 30, and 10 mW/cm²), respectively. In the kinetic analysis experiments, illumination was carried out for 1, 5, 10, 15, and 25 min at a distance of 5 cm, corresponding to an illumination density of 3 × 10⁴ lux (90 mW/cm²). Unless specified, illumination was carried out in a 4°C cold room to prevent over-heating of the photocatalyst substrates and prevent drying. After illumination, the bacterial solutions were recovered from the photocatalyst substrates, and an aliquot of fresh culture medium was used to collect the residual bacteria on the substrates. The two bacterial solutions were pooled to make a total volume of 150 μl. The
bacterial concentration was determined by the standard plating method immediately after the bacterial collection, and the percentage of surviving bacteria was calculated. In spore experiments, $1 \times 10^6$ CFU ($1 \times 10^5$ CFU/ml in 100 µl) were used, and the procedures followed the same protocols as in the live bacteria experiments.

### Mouse model

Six to 8 week-old C57BL/6j mice were purchased from the National Experimental Animal Center (Taipei, Taiwan) [37,43]. The methods used in bacteremia experiments were modified from previous descriptions [9]. Mortality of C57BL/6j mice from anthrax spores treatments ($1 \times 10^6$ to $1 \times 10^7$) was recorded within one week to serve as reference points. To determine the anti-bacterial effect of TiO$_2$ (N), each mouse received an intravenous injection of $1 \times 10^5$ CFU spores of B. anthracis, a lethal dose for mice, with or without pretreatment by photocatalysis on TiO$_2$ (N) or TiO$_2$ substrates ($3 \times 10^4$ lux, 10 minutes at 4°C). In the lethal toxin (LT) experiments, mortality of mice after a lethal dose of LT was performed based on previously described methods [37]. Each mouse received an intravenous injection of LT ($100 \mu$g/g, L:F:PA = 1:5), a lethal dose for mice, with or without pretreatment of photocatalysis on TiO$_2$ (N) or TiO$_2$ substrates ($3 \times 10^4$ lux, 10 minutes at 4°C or 25°C). The mortality of mice was then recorded. During the photocatalysis reaction, the distance between lamps with bacteria- or LT-containing photocatalyst substrates was 5 cm, corresponding to an illumination density of $3 \times 10^4$ lux (or 90 mW/cm$^2$). Relative protein levels of PA and LF in the LT mixtures used in animal experiments were detected by Western blot using rabbit polyclonal anti-PA and anti-LF antibodies, and then probed by secondary supplements was used in this analysis.

### Statistical analysis

All results were calculated from data of at least three independent experiments. A t-test was used to assess the significance of differences in results of anti-microbial effects. A $P$ value of less than 0.05 ($P<0.05$) was considered statistically significant. The statistical tests were carried out and output to graphs using Microsoft Excel (Microsoft Taiwan, Taipei, Taiwan) and SigmaPlot (Systat Software, Point Richmond, CA, USA) software.

### Author Contributions

Conceived and designed the experiments: JHK DSS HHH HHC. Performed the experiments: JHK HHH MSW. Analyzed the data: JHK MSW HHC. Contributed reagents/materials/analysis tools: JHK DSS HHH HHC. Wrote the paper: HHC.
18. Ireland JC, Klostermann P, Rice EW, Clark RM (1993) Inactivation of Escherichia coli by titanium dioxide photocatalytic oxidation. Appl Environ Microbiol 59: 1668–1670.

19. Berkbolet M, Araz CV (1996) Inactivation of Escherichia coli by photocatalytic oxidation. Chemosphere 32: 959–963.

20. Yu JC, Ho W, Yu J, Yip H, Wong PK, et al. (2005) Efficient visible-light-induced photocatalytic disinfection on sulfur-doped nanocrystalline titania. Environ Sci Technol 39: 1173–1179.

21. Hu C, Lou Y, Qiu J, Hu X, Wang A (2006) Ag/AgBr/TiO2 visible light photocatalyst for destruction of azo dyes and bacteria. J Phys Chem B 110: 4066–4072.

22. Shieh KJ, Li M, Lee YH, Sheu SD, Liu YT, et al. (2000) Antibacterial performance of bacterial spores of titanium thin film fabricated by deposition effect in visible light. Nanomedicine 2: 121–126.

23. Elahifard MR, Rahimnejad S, Haghighi S, Gholami MR (2007) Apatite-coated Ag/AgBr/TiO2 visible-light photocatalyst for destruction of bacteria. J Am Chem Soc 129: 9552–9553.

24. Li Q, Xie R, Li YW, Mintz EA, Shang JK (2007) Enhanced visible-light-induced photocatalytic disinfection of E. coli by carbon-sensitized nitrogen-doped titanium oxide. Environ Sci Technol 41: 5050–5056.

25. Cheng CL, Sun DS, Chu WC, Tseng YH, Ho HC, et al. (2009) The effects of the bacterial interaction with visible-light responsive titania photocatalyst on the bactericidal performance. J Biomed Sci 16: In Press.

26. Russell AD (1990) Bacterial spores and chemical sporidical agents. Cln Microbiol Rev 3: 99–119.

27. Driks A (1999) Bacillus subtilis spore coat. Microbiol Mol Biol Rev 63: 1–20.

28. Frouet A, Mock M (2006) Regulatory networks for virulence and persistence of Bacillus anthracis. Curr Opin Microbiol 9: 160–166.

29. Zan L, Lan Y, Qu J, Hu X, Wang A (2006) Ag/AgBr/TiO2 visible-light photocatalyst for destruction of bacteria. J Phys Chem B 109: 1653–1659.

30. Guidi-Rontani C, Levy M, Obayou H, Mock M (2001) Fate of germinated Bacillus anthracis spores in primary murine macrophages. Mol Microbiol 42: 931–938.

31. Guidi-Rontani C, Weber-Levy M, Labbyuere E, Mock M (1999) Germination of Bacillus anthracis spores within alveolar macrophages. Mol Microbiol 31: 9–17.

32. Cote CK, Rossi CA, Kang AS, Morrow PR, Lee JS, et al. (2005) The detection of protective antigen (PA) associated with spores of Bacillus anthracis and the effects of anti-PA antibodies on spore germination and macrophage interactions. Microb Pathog 38: 209–225.

33. Kudva IT, Griffin RW, Garren JM, Calderwood SB, John M (2005) Identification of a protein subset of the anthrax spore immunome in humans immunized with the anthrax vaccine adsorbed preparation. Infect Immun 73: 5685–5696.

34. Yang MC, Yang TS, Weng MS (2004) Nitrogen-doped titanium oxide films as visible light photocatalyst by vapor deposition. Thin Solid Films. pp 1–5.

35. Yang TS, Shiu CB, Weng MS (2004) Structure and hydrophilicy of titanium oxide films prepared by electron beam evaporation. Surface Science 548: 75–82.

36. Kau JH, Lin CG, Huang HH, Hsu HL, Chen KC, et al. (2002) Calyculin A sensitive protein phosphatase is required for Bacillus anthracis lethal toxin induced cytotoxicity. Curr Microbiol 44: 106–111.

37. Kau JH, Sun DS, Tsai WJ, Shyu HF, Huang HH, et al. (2005) Antiplatelet Activities of Anthrax Lethal Toxin Are Associated with Suppressed p42/44 and p38 Mitogen-Activated Protein Kinase Pathways in the Platelets. J Infect Dis 192: 1465–1474.

38. Bizzoni D, Brandelli A (2004) Influence of media and temperature on bacteriocin production by Bacillus cereus R8A during batch cultivation. Appl Microbiol Biotechnol 63: 158–162.

39. Li E, Voutsos AA (1975) Metalloprotease from Bacillus thuringiensis. Appl Microbiol 30: 354–361.

40. Kort R, O’Brien AG, van Stokkum IH, Oomes SJ, Crielaard W, et al. (2005) Assessment of heat resistance of bacterial spores from food product isolates by fluorescence monitoring of dipicolinic acid release. Appl Environ Microbiol 71: 3556–3564.

41. Carrera M, Zandomeni RO, Fitzgibbon J, Sagripanti JL (2007) Difference between the spore sizes of Bacillus anthracis and other Bacillus species. J Appl Microbiol 102: 303–312.

42. Sagripanti JL, Bonifacino A (1996) Comparative sporidical effects of liquid chemical agents. Appl Environ Microbiol 62: 545–551.

43. Sun DS, King CC, Huang HS, Shih YL, Lee CC, et al. (2007) Antiplatelet autoantibodies elicited by dengue virus non-structural protein 1 cause thrombocytopenia and mortality in mice. J Thromb Haemost 5: 2291–2299.

44. Chang JC, Chang HH, Lin CT, Lo SJ (2005) The integrin alphabeta1 modulates P16K and Cdc42 activities induces dynamic filopodium formation in human platelets. J Biomed Sci 12: 881–898.

45. Sun DS, Lo SJ, Lin CH, Yu MS, Huang CY, et al. (2005) Calcium oscillation and phosphatidylinositol 3-kinase positively regulate integrin alphabeta3-mediated outside-in signaling. J Biomed Sci 12: 321–331.

46. Chang HH, Shih KN, Lo SJ (2000) Receptor-mediated endocytosis as a selection force to enrich bacteria expressing rhodostomin on their surface. J Biomed Sci 7: 42–50.

47. Leahy MB, Desens JF, Nuttall PA (1997) Striking conformational similarities between the transcription promoters of Thogoto and influenza A viruses: evidence for intranstrand base pairing in the 5’ promoter arm. J Virol 71: 8352–8356.