The Use of Nanoscale Visible Light-Responsive Photocatalyst TiO$_2$-Pt for the Elimination of Soil-Borne Pathogens

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Abstract

Exposure to the soil-borne pathogens *Burkholderia pseudomallei* and *Burkholderia cenocepacia* can lead to severe infections and even mortality. These pathogens exhibit a high resistance to antibiotic treatments. In addition, no licensed vaccine is currently available. A nanoscale platinum-containing titania photocatalyst (TiO$_2$-Pt) has been shown to have a superior visible light-responsive photocatalytic activity to degrade chemical contaminants like nitrogen oxides. The antibacterial activity of the catalyst and its potential use in soil pathogen control were evaluated. Using the plating method, we found that TiO$_2$-Pt exerts superior antibacterial performance against *Escherichia coli* compared to other commercially available and laboratory prepared ultraviolet/visible light-responsive titania photocatalysts. TiO$_2$-Pt-mediated photocatalysis also affectively eliminates the soil-borne bacteria *B. pseudomallei* and *B. cenocepacia*. An air pouch infection mouse model further revealed that TiO$_2$-Pt-mediated photocatalysis could reduce the pathogenicity of both strains of bacteria. Unexpectedly, water containing up to 10% w/v dissolved soil particles did not reduce the antibacterial potency of TiO$_2$-Pt, suggesting that the TiO$_2$-Pt photocatalyst is suitable for use in soil-contaminated environments. The TiO$_2$-Pt photocatalyst exerted superior antibacterial activity against a broad spectrum of human pathogens, including *B. pseudomallei* and *B. cenocepacia*. Soil particles (<10% w/v) did not significantly reduce the antibacterial activity of TiO$_2$-Pt in water. These findings suggest that the TiO$_2$-Pt photocatalyst may have potential applications in the development of bactericides for soil-borne pathogens.

Citation: Chen Y-L, Chen Y-S, Chan H, Tseng Y-H, Yang S-R, et al. (2012) The Use of Nanoscale Visible Light-Responsive Photocatalyst TiO$_2$-Pt for the Elimination of Soil-Borne Pathogens. PLoS ONE 7(2): e31212. doi:10.1371/journal.pone.0031212

Editor: Dipshikha Chakravortty, Indian Institute of Science, India

Received November 16, 2011; Accepted January 4, 2012; Published February 22, 2012

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Funding: This work is supported by the National Science Council, Taiwan, People’s Republic of China, under grant numbers 95-2314-B-320-009-MY3, 98-2320-B-017-001-MY3 and 99-2320-B-017-002-MY3, by the Ministry of Economic Affairs, Taiwan, People’s Republic of China, under grant number 98-EC-17-A-19-S2-0111 and by Tzu-Chi University under grant numbers TCIRP 95002-02, TCIRP 98001-01, TCRPP 99020 and TCRPP 100003. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

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Introduction

Upon ultraviolet (UV) light illumination, a traditional photocatalyst generates pairs of electrons and holes (electron vacancies in valence bands) to yield reactive oxygen species (ROS) [1], which can oxidize organic substances and kill pathogenic bacteria [2]. Titania photocatalysts have great potential for use in water and sewage treatment because they are stable in water, non-toxic by ingestion and inexpensive [2]. Because the energy source can be solar light, TiO$_2$ photocatalysts are also useful in remote areas where sufficient electricity is not available. To obtain higher quantum efficiencies and reduce the potential exposure of humans and animals to bio-hazardous UV light, ion-doped TiO$_2$ materials with improved visible light responsiveness have recently been developed [3,4]. These photocatalytic materials have different degrees of bactericidal properties [5–10]. Despite their advantages, the elimination of soil-borne pathogens using UV and visible light-responsive photocatalysts has not been clearly addressed.

This study focused on *Burkholderia pseudomallei* and *Burkholderia cenocepacia*, two soil-borne pathogens that can cause the fatal infectious diseases melioidosis and cepacia syndrome, respectively [11,12]. Humans are usually infected with these pathogens through inhalation or cutaneous contact with contaminated soil or water [11,13–16]. These two pathogens are both motile, rod-shaped, Gram-negative bacteria, but they also exhibit certain distinct features. *B. cenocepacia* is widely distributed in the natural environment but is also found in hospitals. This may explain the spread of *B. cenocepacia* that sometimes occurs in intensive care units or oncology wards [14,17,18]. However, *B. cenocepacia* species primarily participate in non-hazardous interactions with plants. Some of these bacteria are even beneficial to humans because they can produce biosurfactants that increase the solubility of pesticides present in polluted environments or provide effective defenses for crop plants against the pathogenic fungus *Fusarium verticillioides* [19,20]. These benefits, however, may increase incidental human contact. By contrast, *B. pseudomallei* mainly dwell in the soil at a...
depth of up to 60 cm below the soil surface. Thus, outbreaks of *B. pseudomallei* usually occur after seasonal events, such as monsoons or typhoons [11,13,21,22], although *B. pseudomallei* still affects millions of people in many countries annually [23,24]. Because *B. pseudomallei* is a potent infectious agent, aerosol exposure during an intentional attack remains a concern [25]. Many clinical isolates of *B. pseudomallei* can tolerate a wild spectrum of antibiotics/ bactericides, including penicillin, first- and second-generation cephalosporins and many of the aminoglycosides [16]. Similarly, *B. cenocepacia* are intrinsically resistant to most clinically relevant antibiotics such as quinolones, aminoglycosides and β-lactam agents, including monobactams and carbapenems [15,26]. Unfortunately, there are still no licensed vaccines available for these pathogens. Because both bacteria steadily adhere to soil particles, resulting in the transmission of disease when the bacteria are aerosolized [12,13] or contaminate soil and water [13,14], effective control strategies to overcome the spread of these bacteria need to be developed.

A visible light-responsive titania photocatalyst is a conceptually feasible candidate for an antibacterial approach because it combines the advantages of a titania photocatalyst in water and sewage treatment [2] with the user-friendliness of visible light [5–7]. To select a high-performing visible light-responsive photocatalyst, commercially available and laboratory-prepared photocatalysts were evaluated for photocatalysis-mediated antibacterial activity against *Escherichia coli*. TiO$_2$-Pt nanoparticles enable superior photocatalytic degradation of pollutant nitrogen oxides [27], and this study demonstrated that, among our selected photocatalysts, TiO$_2$-Pt nanoparticles also exhibit superior antibacterial activity. Therefore, the antibacterial activity of TiO$_2$-Pt nanoparticles against *B. pseudomallei* and *B. cenocepacia* in suspended cultures and/or biofilms was further evaluated under visible light illumination. The attenuation of soil-borne pathogens by TiO$_2$-Pt-mediated photocatalysis was also investigated in a mouse model. We unexpectedly found that the TiO$_2$-Pt photocatalyst continued to exhibit superior antibacterial activity in soil-contaminated water. The potential mechanisms are discussed.

**Results**

**Antibacterial activity of various titania photocatalysts**

Under visible light illumination, the nanoscale TiO$_2$-Pt samples exerted superior killing of *E. coli* [5,10] compared to the commercially available BA-PW25 [28,29] and carbon-containing TiO$_2$ (C150, C200) [7,9,30] (Fig. 1, *P*<0.05, **P*<0.01 and ***P*<0.001, compared to the respective without light groups). The ultraviolet (UV) light-responsive photocatalyst (ST01) was used as a negative control because it does not respond to visible light illumination (Fig. 1, ST01 groups) [7,27]. To further investigate the bactericidal spectrum of TiO$_2$-Pt nanoparticles, various human pathogens, including different strains of the soil-borne bacteria *B. pseudomallei* and *B. cenocepacia*, were analyzed (Table 1). TiO$_2$-Pt-mediated photocatalysis eliminated a wide spectrum of human pathogens. Among these pathogens, *B. pseudomallei* strains were the most susceptible to TiO$_2$-Pt-mediated photocatalysis (Table 1, *B. pseudomallei* vgh07, vgh19, vgh21; 15–18% survival rates).

**Attenuation of *B. pseudomallei* and *B. cenocepacia* by TiO$_2$-Pt-mediated photocatalysis**

In addition to the killing effect, our previous works indicated that photocatalysis introduced cellular damages to those survivors also plays an important role in the attenuation of pathogenic bacteria [6]. Accordingly, we hypothesized that the viable populations of photocatalyzed *B. pseudomallei* vgh07 or *B. cenocepacia* P2 (survival rates of 17% and 26% in Table 1, respectively) would have reduced pathogenic potency. To investigate this possibility, the lethal doses of *B. pseudomallei* vgh07 and *B. cenocepacia* P2 in mice were first determined. A single inoculation of 1×10$^7$ CFU of *B. pseudomallei* vgh07 resulted in 100% mortality (Fig. 2A, 2B, *B. pseudomallei* 10$^5$ CFU groups). By contrast, up to 1×10$^7$ CFU of *B. cenocepacia* P2 was unable to induce mortality in BALB/cJ mice (Fig. 2A). Because small changes in bacterial doses cause a dramatic difference in mortality, *B. pseudomallei* infection in mice should be a more sensitive model than *B. cenocepacia* for investigating photocatalysis-induced attenuation. However, because the range of sublethal doses is narrow (<10$^5$ CFU), the combined effect of photocatalysis and the host immune defenses easily eliminates the injected *B. pseudomallei*, and thus the attenuation of inflammation is not easily observed. Alternatively, clinical features of cepacia syndrome, such as leukocytosis, inflammation and liver necrosis, were clearly reproduced in BALB/cJ mice to a certain extent using a sublethal dose of *B. cenocepacia* (10$^5$ CFU/mouse). Thus, the bacteria *B. pseudomallei* and *B. cenocepacia* were used to investigate the potential attenuating effects on mortality and the inflammatory response, respectively.

In the mouse model, TiO$_2$-Pt-mediated photocatalysis significantly attenuated the lethal dose of *B. pseudomallei* vgh07 treatments (1×10$^5$ CFU; Fig. 2A, 2B, 100% mortality) and resulted in a 100% survival rate for the infected mice (Fig. 2C, TiO$_2$-Pt groups). Notably, the TiO$_2$-Pt-photocatalysis groups in which there was no mortality (Fig. 2C, TiO$_2$-Pt groups) were apparently equivalent to treatment with 1×10$^5$ CFU of *B. pseudomallei* without photocatalysis (Fig. 2A, 2B, *B. pseudomallei*, 10$^5$ groups). According to the survival rate estimated in the bacterial killing experiments (Table 1, 17%, *B. pseudomallei* vgh07 groups), approximately 1.7×10$^5$ CFU of bacterial cells should theoretically remain viable (1×10$^5$ CFU×17% = 1.7×10$^4$ CFU), which is still a lethal dose for BALB/cJ mice (Fig. 2A, 2B, 1×10$^5$ CFU *B. pseudomallei* groups,

![Figure 1. Antibacterial activity of nanoscale TiO$_2$-Pt.](image)
100% mortality). This suggests that the TiO₂-Pt photocatalyzed bacteria are greatly attenuated, similar to our previous study in which anthrax spores were used as a model system [6]. One possible explanation is that the reduction in the pathogenicity of photocatalyzed B. pseudomallei is due to the combination of the reduction in viable cells and the damage to the surviving cells. It has been suggested that, although photocatalysis-induced damages may be repaired in culture, the bacteria may not be recoverable due to the stress of host phagocytic clearance, thus causing differences in in vitro and in vivo analyses [6,9]. Accordingly, it is estimated that, in addition to bacterial killing, at least a one-log reduction in the pathogenic potency of B. pseudomallei can be attributed to bacterial damage (estimated 1.7×10⁴ CFU viable cells vs. 100% survival rates in Fig. 2C, TiO₂-Pt groups, and Fig. 2A, 101 groups).

Melioidosis frequently manifests in the formation of abscesses in soft tissues and internal organs like the liver [11]. Hepatic cellular

| Table 1. The antibacterial spectrum of the nanoscale TiO₂-Pt photocatalyst. |
|-----------------|-----------------|
| **Species and strains** | **Survival (%)** |
| **Nonpathogenic bacteria** | |
| Escherichia coli | OP50 9±4 |
| **Pathogenic bacteria** | |
| Burkholderia cenocepacia | P2 26±3 |
| | 348 47±2 |
| | BC14 60±1 |
| Burkholderia pseudomallei | vgh07 17±8 |
| | Vgh19 15±4 |
| | Vgh21 18±3 |
| **Soil-borne** | |
| Staphylococcus aureus | ATCC6538P 30±5 |
| | Multidrug resistant, strain 27 45±1 |
| | Multidrug resistant, strain 69 51±7 |
| | Coagulase negative, strain 41 24±5 |
| Acinetobacter baumannii | nknu11 30±4 |
| | Multidrug resistant, strain 49 40±4 |
| **Non-soil-borne** | |
| Legionella pneumophila | ATCC33152 37±3 |
| Pseudomonas aeruginosa | FY32 48±7 |
| Klebsiella pneumoniae | nknu24 34±2 |
| Salmonella typhimurium | FY48 41±7 |

The survival rate of various bacteria after challenged with TiO₂-Pt-mediated photocatalysis. The visible light-driven antibacterial activity of TiO₂-Pt-mediated photocatalysis against various bacteria is shown; the soil-borne pathogens B. pseudomallei and B. cenocepacia were compared with nonpathogenic E. coli OP50 and the pathogenic bacteria S. aureus, A. baumannii, L. pneumophila, P. aeruginosa, K. pneumoniae and S. typhimurium. The untreated groups (without TiO₂-Pt and illumination) of each experiment were normalized to 100%. n = 6, three experiments with 2 replicates). doi:10.1371/journal.pone.0031212.t001

Figure 2. Mortality of mice receiving inoculations of B. pseudomallei with or without photocatalysis. The survival rate of mice receiving inoculations of various doses (10¹–10⁶) of B. pseudomallei vgh07 and B. cenocepacia P2 cells is shown. n = 7, three experiments with 2 or 3 replicates (A). The survival rate and time course of mice treated with 10³–10⁶ CFU of B. pseudomallei vgh07. n = 7, three experiments with 2 or 3 replicates (B). The survival rate and time course of mice treated with 10³ CFU of B. pseudomallei vgh07 with or without TiO₂-Pt-mediated photocatalysis. n = 6, three experiments with 2 replicates (C). doi:10.1371/journal.pone.0031212.g002
debris in the liver has been reproduced in mice that have received an intravascular infection of \textit{B. pseudomallei} [31]. Therefore, in this study, the liver function of mice served as an indicator of disease severity and was evaluated by analyzing the plasma levels of the hepatocyte enzymes aspartate aminotransferase/alanine aminotransferase (AST/ALT), which are markers of liver function (Fig. 3A). In agreement with the mortality data (Fig. 2C), the induction of plasma AST/ALT was significantly reduced when BALB/cJ mice were infected with photocatalyzed \textit{B. pseudomallei} vgh07 (Fig. 3A, TiO$_2$-Pt $\pm$ light vs. light groups, ** $P<0.01$).

Similarly, lesions with cellular debris in the liver were not found in these photocatalyzed groups (Fig. 3B vs. 3C; white arrows: hemorrhage lesions; black arrow: cellular fragmentations and debris [31]).

**Figure 3. Liver damage in mice infected with \textit{B. pseudomallei}**. Twenty-four hours after inoculation with \textit{B. pseudomallei} with or without photocatalysis, the serum AST/ALT levels of mice were examined. The means of the respective groups are indicated as horizontal bars; ** $P<0.01$ and +++ $P<0.001$, compared with the TiO$_2$-Pt+light photocatalyzed groups and without infection groups, respectively (A). The hematoxylin and eosin staining of liver sections from mice treated with \textit{B. pseudomallei} with (B) or without (C) photocatalysis. White arrows: hemorrhage lesions; black arrow: cellular fragment and debris. n = 6, three experiments with 2 replicates.

doi:10.1371/journal.pone.0031212.g003

\textbf{Attenuation of \textit{B. cenocepacia}-mediated inflammation by photocatalysis}

To investigate whether photocatalysis could attenuate \textit{B. cenocepacia} and thus result in reduced inflammation \textit{in vivo}, an air pouch infection mouse was established based on a previously described method [32]. \textit{B. cenocepacia} cells (strains P2 and BC14; $1 \times 10^5$ CFU) were treated with or without TiO$_2$-Pt-mediated photocatalysis (visible light, $1 \times 10^4$ lux) and then injected into air pouches underneath the skin of mice (Fig. 4A, the experiment outline). Twenty-four hours post treatment, viable bacteria were recovered from the air pouch (Fig. 4A, experiment outline). In agreement with the \textit{in vitro} analysis (Table 1, P2 and BC14 groups), photocatalysis was associated with significantly fewer viable bacteria (Fig. 4B, ** $P<0.01$, compared with the respective light-only/without TiO$_2$-Pt groups).
Forty-eight hours after the injection of *B. cenocepacia* P2 and BC14, which allowed the infection to become established in the internal organs, the serum levels of the cytokines interleukin-1 (IL-1) and tumor necrosis factor-α (TNF-α) were characterized (Fig. 4A, experiment outline; Fig. 4C–D). In agreement with the analyses performed in vitro (Table 1, P2 and BC14 groups) and in vivo (Fig. 4B), the photocatalyzed *B. cenocepacia* caused a significantly lower induction of IL-1 and TNF-α than bacteria that were not treated with photocatalysis (Fig. 4C, 4D; light vs. light+TiO2-Pt groups; *P*<0.05, **P**<0.01). These results suggest that photocatalysis reduced *B. cenocepacia*-mediated inflammatory responses in mice.

Photocatalysis resistance associated with biofilm mass

*B. cenocepacia* was relatively more resistant to photocatalysis than *B. pseudomallei* (Table 1). Photocatalysis-resistant bacteria have not been clearly characterized. The broad resistance spectrum of *B. cenocepacia* isolates may be useful for investigating the mechanism underlying photocatalytic resistance. Previous reports have indicated that biofilm formation is associated with bacterial resistance to antibiotics and the ROS hydrogen peroxide [33–37]. Consequently, bacterial killing experiments were performed with *B. cenocepacia* on biofilms. Photocatalysis tended to increase the survival rate of photocatalyzed *B. cenocepacia* cells (Fig. 5A, P2, 40% survived>Table 1, P2, 26% survived). To further investigate whether the ability to form a biofilm is associated with photocatalysis resistance, the survival rates of the photocatalyzed bacteria (including a total of 33 *B. cenocepacia* isolates, in which the P2 and BC14 data were equivalent to Table 1; the Y-axes of Fig. 5B and Fig. 5C) were plotted against the relative masses of the biofilms (quantified in optical units, the X-axis of Fig. 5B) and the released levels of lipopolysaccharide (LPS) (an indicator that is associated with biofilm formation ability [38]; the X-axis of Fig. 5C). These results suggest that biofilm formation is somewhat associated with resistance to photocatalysis (Fig. 5B–C), although the detailed mechanism remains to be investigated further.

### Scanning electron microscopy

Previous studies have suggested that photocatalysis-induced damage is crucial for the attenuation of bacterial cells [6]. Scanning electron microscopy was used to determine whether photocatalysis induced different deformations of *B. cenocepacia* strains that exhibited different degrees of resistance to photocatalysis (Fig. 6, P2 vs. BC14; P2: A–D; BC14: E–H). The bacteria were treated with (Fig. 6 A, C, E, G) or without (Fig. 6 B, D, F, H) photocatalysis. Cellular deformations were observed in the groups with photocatalysis, indicating that the bacteria were damaged (Fig. 6 A, C, E, G; arrows in C and G). Intriguingly, the P2 strain of *B. cenocepacia* was mainly present as planktonic cells (Fig. 6, A–D). By contrast, BC14 cells mainly gathered in cell clusters, which resembled miniature biofilms (Fig. 6E–H, the small inserts in E and F are the respective low magnification views). This is the first SEM observation of biofilm-like cell clusters of *B. cenocepacia* in a suspended liquid culture. Given that biofilms can resist various bactericides, including ROS [33–37], and that BC14 cells have a stronger tendency to form biofilms than P2 cells (Fig. 5B–C, BC14 vs. P2; Fig. 6E–H vs. 6A–D), it is reasonable to conclude that BC14 cells have a higher resistance to photocatalysis than P2 cells (Table 1, BC14 vs. P2).

### Antibacterial performance of TiO2-Pt in soil-containing solutions

One practical problem in the elimination of soil-borne pathogens is the potential light-shading effect of contaminated...
soil particles. Contaminants such as dye (bromophenol blue) and protein (bovine serum albumin) have been shown to greatly reduce the antibacterial activity of a photocatalyst [5]. To investigate the potential light-shading effect of soil particles, photocatalysis-mediated killing in a soil-containing solution was compared with photocatalysis-mediated killing in solutions of various concentrations of bromophenol blue (Fig. 7A). Unexpectedly, soil particle contaminants up to a concentration of 10% w/v did not significantly influence the elimination of *B. cenocepacia* P2 cells, compared to the strong blocking effect of bromophenol blue at the same dose (10% w/v; Fig. 7A, **P<0.01, compared with the respective bromophenol blue groups). Similar results were also obtained using another *B. cenocepacia* strain (BC14) and *B. pseudomallei* (data not shown). One possibility is that bromophenol blue, but not the soil solution, blocks certain ranges of visible light that are vital for the activation of TiO2-Pt. The UV-visible light absorption spectrum of photocatalyst was therefore examined; the light absorption of TiO2-Pt but not pristine TiO2-Pt covers the entire visible light range (Fig. 7B, TiO2-Pt vs. pristine TiO2-Pt). To investigate whether the different antibacterial outcomes were due to the distinct light-absorbing properties of soil particles and bromophenol blue, the UV-visible absorption spectrum of each solution was determined (Fig. 7C, the concentration of both solutions was 10% w/v). The results indicated that there were only small differences between the light-absorbing properties of the two solutions (Fig. 7C), suggesting that the dramatically different antibacterial outcomes were not primarily due to differences in light absorption.

Another explanation is that the soil particles used in this study might affect bacterial survival. Because soil is the natural habitat of *B. cenocepacia*, soil particles would not be expected to inhibit the bacteria. Nonetheless, to test the specific soil that we used, the growth constants for *B. cenocepacia* were determined in various culture conditions, including one with soil-particle supplements (Table 2). As expected, soil particles did not suppress the growth of *B. cenocepacia* (Table 2, soil media vs. LB and BCS media). Collectively, these results suggest that TiO2-Pt is useful for eliminating pathogens in soil-contaminated water. The differential blocking of photocatalysis by bromophenol blue and soil-containing solutions is interesting, and its mechanism remains to be further investigated.

**Discussion**

Traditional UV-responsive photocatalysts have shown great potential in the development of a new generation of disinfectants [1]. Because UV light can damage human eyes and skin [39,40], photocatalysts with improved visible light responsiveness have been developed [3–7]. The antibacterial activity of a visible light-responsive TiO2-Pt photocatalyst was therefore evaluated in this study. The TiO2-Pt nanoparticles exhibited superior antibacterial activity compared to other commercial and laboratory-prepared photocatalysts in the *E. coli* experiments (Fig. 1). However, a major challenge is that soil pathogens can form biofilms, which can greatly reduce the antibacterial effect of some bactericides [33–35]. UV-responsive photocatalysts have been shown to eliminate the bacteria within a biofilm and suppress biofilm formation [41,42]. However, whether a biofilm contributes to resistance against photocatalysis has not yet been ascertained. The degree to which visible light-responsive photocatalysts eliminate biofilm bacteria is also unclear. Using soil bacteria with differential biofilm-forming abilities, our data clearly demonstrate that biofilm formation is associated with resistance to photocatalysis (Table 1, Fig. 5–6, Fig. 6). The physical barrier created by a biofilm contributes to resistance against bactericides. The extracellular polysaccharide matrices of biofilms can restrict the diffusion and binding of soluble substances to the target bacteria, and thus the
bacteria within the biofilm are protected [33]. This type of protection effectively resists the ROS hydrogen peroxide [36,37]. Biofilm formation may play a more important role than catalase expression in resistance to H$_2$O$_2$ [36,37]. A similar mechanism may be responsible for the resistance to the TiO$_2$-Pt-photocatalysis-produced ROS in this study. Despite this protection, the TiO$_2$-Pt photocatalyst still efficiently attenuated the pathogens. Our mouse model revealed that the pathogenic potency of B. pseudomallei was greatly reduced after photocatalysis (Fig. 2). Because the injection dose of photocatalyzed cells was $1 \times 10^6$ CFU/mouse and ultimately no mortality occurred after the injections (mortality occurred $>1 \times 10^6$ CFU/mouse; Fig. 2A and 2B, $1 \times 10^3$ CFU groups; Fig. 2C, TiO$_2$-Pt vs. no TiO$_2$-Pt groups), it was estimated that at least a 2-log reduction in CFU was achieved ($1 \times 10^6$ to $<1 \times 10^4$ CFU). This is in agreement with our previous study that used photocatalyzed B. anthracis as a model [6].

In both systems, the plating method revealed a less than one-log reduction in viable cells (Table 1), while the in vivo experiments showed an additional reduction over one log (Fig. 2) [6]. Recently, photocatalysis was found to induce deformation and protein leakage in bacterial cells [9]. This damage is crucial for the antibacterial outcome; however, bacterial cells may be able to repair this damage and deformation [9]. Our results suggest that photocatalysis likely induced temporary, repairable damage to the bacteria, which could be recovered on the culture dishes (Table 1, B. pseudomallei vgh07 groups, 17% survived) but could not be recovered under phagocytic clearance in vivo (Fig. 2C, no mortality of TiO$_2$-Pt groups, suggesting the survived bacteria $<10^4$ CFU).

In summary, visible light-responsive TiO$_2$-Pt-mediated photocatalysis successfully attenuated soil-borne pathogens. In addition,
the mouse mortality assay appears to be a relatively sensitive method for detecting sublethal damage to bacteria, compared to the traditional plating method.

An intriguing feature of the TiO$_2$-Pt photocatalyst is its antibacterial performance in soil-contaminated water. When compared with bromophenol blue, soil contaminants seem to have only a limited effect on the inhibition of antibacterial photocatalysis (Fig. 7). This phenomenon has not been previously described. Soil particles contain various components with different surface-binding properties [43,44]. Because photocatalyst-bacteria binding is critical for the antibacterial outcome of photocatalysis [7], soil particles may serve as bridging materials to link the bacteria and the TiO$_2$-Pt nanoparticles and thus facilitate killing. However, a large amount of soil particles prohibits the filming of bacteria-photocatalyst aggregates. Moreover, soil can be penetrated by light to a certain extent, through which the seeds of certain plants are revived by light induction in particular seasons [45,46]. Through pathways like these, the antibacterial performance of TiO$_2$-Pt might be preserved. By contrast, bromophenol blue has been used widely in fluorescence quenching techniques in which fluorescent signals are greatly diminished by the addition of a dye [47,48]. Such a mechanism might reduce the amount of light that reaches TiO$_2$-Pt surfaces. Thus, bromophenol blue and soil particles likely have intrinsic differences in light absorption, scattering and reflection properties. The detailed mechanism underlining the differences in blocking of photocatalysis by dye and soil solutions is an interesting phenomenon that merits further investigation.

In summary, this study is the first to report that biofilm formation is associated with bacterial resistance to photocatalysis-mediated killing. Accordingly, biofilm formation may need careful evaluation and attention if photocatalysts are used to eliminate bacteria in biofilms. Nonetheless, nanoscale TiO$_2$-Pt is an effective bactericide for eliminating soil-borne pathogens. The antibacterial effect significantly reduced the number of viable bacteria and damaged the residual surviving cells, achieving a greater than 2-log reduction in their estimated pathogenic potency. In addition, soil contaminants up to 10% w/v did not significantly reduce the antibacterial performance of TiO$_2$-Pt. Titania photocatalysts have previously been applied as a suspension in a slurry UV reactor, as a thin film coated on a reactor surface or as a membrane filter [2], suggesting that TiO$_2$ photocatalysts may be useful in a variety of settings to reduce the transmission of pathogens in public environments. Because specific methods to control the spread of these bacteria are still lacking, visible-light responsive TiO$_2$-Pt photocatalysts may have potential applications in the development of effective antibacterial strategies against these soil-borne pathogens.

Materials and Methods

Ethics Statement

The animal methods in this study were approved by the Institutional Animal Care and Use Committee at the National Kaohsiung Normal University, Taiwan (approval ID: 9801 and 9901), and the experiments were performed in accordance with the institutional guidelines. These approvals certified the studies of the bactericidal activity of synthesized antibacterial compounds on the elimination of human pathogens, the air pouch infection mouse model and the pathophysiological observation of mouse sera or tissues after infection with *B. pseudomallei* and *B. cenocepacia*.

Preparation of photocatalysts

Platinum-containing nano-structured TiO$_2$ particles (TiO$_2$-Pt) were prepared by the photoreduction process using chloroplatinic acid (H$_2$PtCl$_6$) and commercial TiO$_2$ nanoparticles (Ishihara ST01) as a platinum precursor and a pristine photocatalyst, respectively. TiO$_2$-Pt was prepared by mixing 3 g nonporous TiO$_2$ (ST01) and 97 mg H$_2$PtCl$_6$·6H$_2$O in 100 mL of double-distilled water. The TiO$_2$ suspension and the H$_2$PtCl$_6$ solution were mixed well by ultrasonic treatment for 30 minutes. The initial pH value was adjusted to 4 with 0.1 M NaOH. A nitrogen
Table 2. The specific growth rate constant (k) of B. cenocepacia P2 under various culture conditions.

| Culture media  | Exp 1 | Exp 2 | Exp 3 |
|----------------|-------|-------|-------|
| LB broth       | 0.67  | 0.61  | 0.66  |
| Soil media     | 0.60  | 0.62  | 0.64  |
| BCS media      | 0.66  | 0.64  | 0.61  |

The specific growth rate constant (k) was measured after growing B. cenocepacia P2 in LB broth, soil medium (soil particles 10% w/v in LB) and B. cepacia selective (BCS) medium.

doi:10.1371/journal.pone.0031212.t002

stream at a rate of 100 mL/minute was continuously purged into the reaction chamber to remove oxygen in the solution. The solution was then irradiated with an UVC lamp (TUV 10W/G10 T8, Philips Taiwan, Taipei, Taiwan) with an intensity of 0.7 mW/cm² for 4 hours. Platinum ions were reduced to platinum metallic nanoparticles by the photo-generated electrons of TiO₂ and then deposited onto the surfaces of the TiO₂. TiO₂-Pt nanoparticles with a Pt/Ti molar ratio of 0.5% were obtained by centrifuging at 1×10³ rpm, washing with DI water and then drying at 375 K for 3 hours.

Bacterial strains and culturing

Staphylococcus aureus BCRC10451, Escherichia coli BCRC11634, Legionella pneumophila ATCC33152, Klebsiella pneumonia nkm24 and Salmonella typhimurium FY148 were kindly obtained from the Center for Environmental Services (National Kaohsiung Normal University, Taiwan). Burkholderia pseudomallei (vgh07, vgh19 and vgh21), Pseudomonas aeruginosa FY32 and multidrug-resistant (Strain 49) and drug-susceptible Acinetobacter baumannii nku11 were received from the Kaohsiung Veterans General Hospital (KVGH, Taiwan); their characteristics have been described in previous studies [49–51]. Burkholderia cenocepacia BC14 and other Staphylococcus strains (Strain 27, 41 and 69) were isolated from patients with septicemia in KVGH, Taiwan. All of the other B. cenocepacia strains (32 different isolates), including P2 and 34B, were isolated from the soil at the Er-Ren River Basin and the countryside of Kaohsiung County, Taiwan. All of the strains were confirmed by an automatic system (BD Phoenix 100 Automated Microbiology System, Becton, Dickinson and Company, Franklin Lakes, NJ) and, if necessary, by the nucleotide sequences of their 16S rRNA gene sequences. To avoid the light shedding effect, none of the B. cenocepacia strains used were pigment producers. Luria-Bertani (LB) broth was used to culture all of the bacteria.

Detection of viable bacteria in suspensions or biofilms after photocatalysis

Bacterial concentrations were determined by the standard plating method [32,53] and inferred from optical density readings at 600 nm (OD₆₀₀). A factor for converting the OD₆₀₀ values of the bacterial culture to concentration (colony forming units [CFU]/mL) was calculated as follows. A fresh bacterial culture was diluted by factors of 10⁻¹ to 10⁻⁷, and the OD₆₀₀ of these dilutions was measured. The 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 particular bacteria were calculated. The relative amount of viable bacteria estimated using this calculation was confirmed by the plating method.

To determine the bactericidal effects of the photocatalysts, 200 μL overnight bacterial culture was transferred into 5 mL culture medium and incubated at 37°C until an OD₆₀₀ of 0.3 to 0.6 (log phase) was achieved. The bacterial concentrations were calculated using the conversion factor for the bacteria. Aliquots of 1×10⁵ CFU bacteria were mixed with TiO₂-Pt nanoparticles (50 μg/mL) using a plastic yellow tip and placed onto a 24-well plate. For the photocatalytic reaction, the 24-well plates containing bacteria were then placed under an incandescent lamp (Classic tone incandescent lamp, 60W, Philips Taiwan, Taipei, Taiwan); no UV range emissions of incident light were present. A light meter (LX-102, Lutron Electronic Enterprises, Taipei, Taiwan) was used to measure the illumination density.

To compare the photocatalysis activity of TiO₂-Pt with other photocatalysts, illuminations were performed with an illumination density of 1×10⁴ lux for 40 minutes. After illumination, the bacterial solutions were recovered, and an aliquot of fresh culture medium was used to collect the residual bacteria from the wells. These two bacterial solutions were pooled together. The bacterial concentration was determined by the standard plating method immediately after bacterial collection, and the percentage of surviving bacteria was calculated. A commercially available UV-responsive photocatalyst, ST01 (also the pristine photocatalyst), and a UV/Vis-responsive photocatalyst, BA-PW25 (Ecodevice, Tokyo, Japan) [28,29], were used as comparisons.

Biofilm formation was performed on 96-well multiwell plates [54]. Suspensions of B. cenocepacia or B. pseudomallei (10⁵ CFU/mL; 100 μL) in log phase were seeded into polystyrene 96-well plates (BD Falcon, Erembodegem, Belgium). Four hours post-adhesion, the non-adhered cells were removed using 100 μL normal saline (0.9% w/v NaCl). After 3 washes, the photocatalytic reaction was conducted in the 96-well plates, which contained adherent bacterial cells (approximately 1×10⁶ CFU) and 20 μg TiO₂-Pt in 0.5 mL saline, with illumination with visible light with a density of 4×10⁴ lux. To measure the relative masses of the respective biofilms, 100 μL fresh LB broth was added to each well after washing with normal saline in another plate. After incubation for 20 hours, the supernatant was again removed, and the wells were washed with 100 μL saline solution. In the crystal violet assay, 100 μL 99% methanol was added and incubated for 15 minutes to fix the biofilms, after which the supernatants were removed and the plates were air-dried. Next, 100 μL crystal violet solution (0.1%, Pro-Lab Diagnostics, Richmond Hill, ON, Canada) was added to each well. After 20 minutes at room temperature, the excess crystal violet was removed by washing the plates under running tap water. Finally, the bound crystal violet was released by adding 150 μL 33% acetic acid (Sigma-Aldrich, St. Louis, MO). The absorbance was measured at 590 nm using a multilabel microtiter plate reader (Wallac Victor; Perkin Elmer Life and Analytical Sciences, Boston, MA).
Mouse mortality analysis
To determine the lethal doses of *B. pseudomallei* vgh07 and *B. cenocepacia* P2, the bacteria were grown to log phase (OD$_{600}$ of 0.5–0.6). After 3 washes and resuspension with normal saline, the bacteria solutions were diluted to various concentrations ($1 \times 10^2$ to $1 \times 10^6$ CFU in 0.5 mL saline). BALB/cj mice then received intravascular treatments with these bacteria ($1 \times 10^1$ to $1 \times 10^5$ CFU/mouse), and their mortality was recorded every day for 6 days. To evaluate photocatalysis-mediated attenuation, $1 \times 10^5$ CFU *B. pseudomallei* (log phase) were treated with or without photocatalysis (20 μg TiO$_2$-Pt, $1 \times 10^4$ lux, 40 minutes, in 100 μL saline). The mortality was recorded after the mice were treated with the bacteria intravascularly.

Hematoxylin and eosin (H & E) staining
After treatment with the photocatalyzed bacteria for 24 hours, livers were excised from the infected mice, fixed in 10% formalin, embedded in paraffin, sectioned (4 μm) and stained with hematoxylin and eosin. The images of necrosis and cellular debris in the liver tissue were observed in 30 high-power fields (400×) under a microscope (DMI20E; Leica, Wetzlar, Germany).

Analysis of liver function
To analyze liver function, whole blood samples (50–100 μL) were collected from mice hearts and mixed with anticoagulant solution (0.1 M sodium citrate) in Eppendorf tubes. The levels of aspartate aminotransferase/alanine aminotransferase (AST/ALT) were measured with a clinical biochemistry analysis system (COBAS INTEGRA® 800, Roche Taiwan, Taipei, Taiwan) at 24 hours post-infection.

Detection of LPS
The concentration of 3-hydroxytetradecanoic acid (C14:0 3-OH FA), a surrogate for LPS, was measured with an Agilent 6890 gas chromatograph/5973N mass selective detector (GC-MS) system [55]. Briefly, the 7-day-cultures of each strain were filtered (0.45 μm), lyophilized and resuspended in 1 mL methanolic NaOH (3.8N). This methanolic solution was heated to 100°C in a cooled counterflow system for 30 minutes and then adjusted with methanolic HCl (2.5N) at 80°C for another 10 minutes. After esterification, hexane was added as a partition solution in a proportion of 1:1 for 5 minutes. The upper organic aqueous layer was removed, dried with nitrogen gas and resuspended using 1 mL of hexane.

The GC was equipped with a 60-m DB23-MS (Andover, MA) capillary column (0.25-mm ID; 0.25-μm film thickness). The injector and interface temperatures were maintained at 260°C and 280°C, respectively. The oven temperature was held at 110°C for 1 minute, and then a two-step program was used to increase the temperature to 175°C at 25°C/minute and then to 220°C at 1.5°C/minute. Finally, the temperature was held at 220°C for 3 minutes. The following parameters were used for injecting the samples into the GC/MSD system: sample size, 1 μL; injection mode, splitless; injector purge-off duration, 1 minute; solvent delay, 5.9 minutes.

The mass range adopted for the collection of the full-scan mass spectra was m/z 50–550. Based on the ion intensity data from the full-scan mass spectra, the differentially fragmented ions (43/103/166 m/z) were then used for identification and quantification. The amount of 3-OH FA present, after adjustment relative to the recovered internal standard concentration, was plotted against the ratio of the areas of the tested C14:0 3-OH FA and the areas of the standards.

Detection of cytokines
TNF-α and IL-1 levels in the mouse sera were analyzed using ELISA kits (Diaclone Inc., Besancon Cedex, France) according to the manufacturer’s instructions.

Scanning electron microscopic (SEM) imaging
The SEM analysis was performed as previously described [56]. Photocatalyzed bacteria were incubated on bovine serum albumin-precoated (1% w/v) cover slides for 15 minutes. Bacteria that were attached to the cover slides were then fixed with glutaraldehyde and subjected to alcohol dehydration, critical point drying procedures, and gold coating [57] and observed under a scanning electron microscope at 15 kV (S-4700, Hitachi High-Technologies, Tokyo, Japan). At least three different areas were randomly selected for photography at each magnification; representative data are shown.

Air pouch infection mouse model
BALB/cj mice (males, 8–10 weeks of age) were purchased from the National Laboratory Animal Center (NLAC, Taipei, Taiwan). The mice were housed in the Laboratory Animal Center of National Kaohsiung Normal University (Kaohsiung, Taiwan). The air pouch infection was modified based on a previously described approach [8,32]. After anesthesia and shaving of the hairs around the injection sites, the BALB/cj mice were subcutaneously injected with 1 mL air to form an air pouch. Suspensions of *B. cenocepacia* (log phase, 10$^5$ CFU, 0.2 mL phosphate buffered saline [PBS]) with or without photocatalysis were injected into the air pouches. At 48 hours after photocatalytic treatment, the air pouches were injected with an additional 1 mL PBS. The bacteria-containing solution (1 mL) was then collected from the air pouch. The number of surviving bacteria (CFU) was determined using the standard plating method.

Photocatalysis in bromophenol blue- and soil-containing solutions
The soil samples were obtained from the Er-Ren River Basin in southern Taiwan, which features vigorously growing vegetation and is a natural habitat for *B. pseudomallei* and *B. cenocepacia* [22]. After dissolution in distilled water, the large particles were removed using a one-mm sieve. The remaining small particles were sterilized using an autoclave. After sedimentation by centrifugation at 600 g for 10 minutes, the soil particles were resuspended in normal saline. Bacteria (1 × 10$^5$ CFU) and the photocatalyst-containing saline (20 μg TiO$_2$-Pt) solutions were supplemented by various concentrations of bromophenol blue (Sigma-Aldrich) or soil particles to a final volume of 0.5 mL saline solution. Photocatalysis was performed in 24-well dishes with illumination of visible light at a density of 4 × 10$^4$ lux. The viable bacteria were then determined using the plating method.

Determination of the specific growth rate constant of *B. cenocepacia* P2
*B. cenocepacia* P2 was grown in LB, BCS (*B. cepacia* selective) or soil media. The specific growth constant (K) was calculated during the first 3 to 5 hours of growth at 37°C with the equation lnN = lnN$_0$ = K (t-t$_0$) and was derived from the mean of triplicate experiments. N represents the cell concentration per mL of a five-hour culture (t); N$_0$ represents the cell concentration of a three-hour culture (t$_0$) [58].
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