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Photoinduced Bactericidal Effect of Titania Thin Film against *Legionella pneumophila*

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Abstract：In this study, the photoinduced bactericidal or disinfectant effect of titanium dioxide (TiO_2) based films against *Legionella pneumophila* (*L. pneumophila*) was examined. The *Legionella* species are environmentally abundant bacteria isolated from water and soil, and they are increasingly recognized as a cause of severe pneumonia for elderly people. The titania (TiO_2) thin film photocatalyst was prepared by dip coating on a glass plate. The bactericidal effect was evaluated by the observation of the bacterial cell walls using a scanning electron microscope (SEM), and by the degradation of leaking endotoxin, as well as by the bacterial survival rate. When the TiO_2 photocatalyst was exposed to ultraviolet irradiation (UV–A or UV–C), it showed a strong bactericidal effect on *L. pneumophila*, which was confirmed by the destruction and dissolution of the bacterial cell walls through the SEM. UV irradiation without TiO_2 photocatalyst also caused bacterial inactivation and disruption leading to the leakage of endotoxin. However, the complete degradation of leaking endotoxin was only observed when the titania thin film was exposed to the UV irradiation. Furthermore, the disruption and dissolution of the cell wall were shown by the use of TiO_2 photocatalyst with UV irradiation, however, between the UV rays, UV–C caused a bactericidal effect earlier and more profoundly than UV–A. Although the generally used disinfectants, such as hydrogen peroxide and hypochlorous acid, are able to sterilize bacteria, they did not show a complete degradation effect for endotoxin. Consequently, in this study, the antibacterial effect of TiO_2 thin film photocatalyst with UV irradiation was proven by showing the abilities of the bacterial inactivation and disruption and the degradation or detoxification of endotoxin. It was also suggested that the use of TiO_2 photocatalysts as a disinfectant would be a safer and cleaner alternative technology for environmental protection against microbial pollution than other chemical disinfectants.

Key words：TiO_2 photocatalyst, Disinfectant, *Legionella pneumophila*, Endotoxin

Introduction

The genus *Legionella*, Gram–negative micro–aerophilic bacteria, are environmentally abundant ones that can be isolated from water and soil samples. Legionellosis, an infection caused by a number of *Legionella* species, can range from mild respiratory illness to severe life–threatening pneumonia, and the majority of its cases are caused by the *L. pneu-*

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mophila serogroup.[1] Since the first outbreak of legionellosis as "Legionnaires' disease" in a Philadelphia hotel in 1977,[2] contaminated building water-distribution systems including the cooling tower[3)4] and other aerosol-producing devices[5)6] have been recognized to cause outbreaks of this illness. However, in Japan, hot springs and public baths are more probable sources of the legionellosis.[7-9] In general, chemical disinfectants including chlorinated compounds such as chlorite are used to prevent microbial contaminations in these facilities. However, the widespread use of these chemicals gives rise to other types of environmental pollution, as well as the emergence of more resistant and virulent strains of microorganisms. There is thus an immediate need to develop alternative sterilization technologies.

Titanium dioxide (TiO_2) is an anatase form semiconductor. Since the discovery of photoinduced water cleavage on TiO_2 electrodes,[10] many studies have been done on TiO_2 photocatalysis with the aim of developing methods to purify or sterilize water and air.[11-14] In addition, the killing of cancer cells with the TiO_2 photocatalyst for medical applications has also been reported.[15,16] In the earlier stages, the powdered TiO_2 preparations were suggested to be useful for the solution of water pollution caused by indecomposable organic compounds and for the sterilization of water.[17-19] One of the main problems, however, has been the fact that the process of separating the powder from water is often troublesome. Therefore, efforts were made to fix or immobilize the TiO_2 powder onto the surface of solid materials.[20-23]

In our laboratory, a thin film of TiO_2 on a glass plate was successfully prepared, and it was revealed that this TiO_2 photocatalyst was resistant to acid and had the ability to degrade dinitrophenol under black light irradiation.[24,25] We also tested its virus-inactivating effects using *Lactobacillus* PL-1 phages as a model.[26] In the present study, we examined whether the TiO_2 thin film photocatalyst that was developed in our laboratory has, under UV irradiation, any bactericidal activity against *L. pneumophila* in an aqueous solution, as well as the effective detoxification (degradation) ability of the exhausting endotoxin from the dead bacteria. Endotoxin is the lipopolysaccharide (LPS) of cell wall constituents of Gram-negative bacteria, which consists of a sugar chain expressed O-antigen and a complex lipid called lipid-A. Lipid-A plays a major role in the various types of bioactivity of endotoxin, including such endotoxic activities as pyrogenicity and lethal toxicity. The problem is that such endotoxic activities are elicited even at very low concentrations; i.e., a few nanograms per milliliter of endotoxin can cause critical problems in medical facilities and in factories manufacturing pharmaceuticals and medical devices. As a result, if the TiO_2 photocatalyst can degrade endotoxin, then it may be a potentially useful modality for the prevention of microbial pollution.

Materials and Methods

Bacterial strain:

A strain of *L. pneumophila* (serogroup 1) was donated by Pr. Yoshida of Kyushu University (Department of Microbiology, Faculty of Medical Science). This strain was cultured on buffered charcoal yeast extract (BCYE) agar plates (Eiken Chemical Co., Tokyo, Japan) at 37℃ for at least 3 days. The bacteria were then suspended in sterile PBS and adjusted to 10^6 CFU/ml in PBS.

Preparation of TiO_2 thin film:

The fine powder of amorphous titanium dioxide, obtained by a successive reaction of hydrolysis and polymerization of titanium tetra-*iso*-propoxide (Sigma Aldrich Japan, Tokyo, Japan), was dissolved in 31% hydrogen peroxide solution, and the mixture was stirred at 10℃ for 2 h. A transparent yellow gel was formed with the generation of oxygen bubbles. The gel was then treated again with hydrogen peroxide under the same conditions for 30 min to form a transparent sol without bubbles. A glass plate (55×40×1 mm), which had been ultrasonically cleaned with isopropanol, was dip-coated with the sol, and was dried carefully at room temperature in a clean bench, and then was calcined at 500℃ for 5 h. The thin film was identified to be an anatase as reported previously.[24]

Disinfectants :

As generally used disinfectants, hydrogen peroxide (H_2O_2 ; Wako Pure Chemical Industries, Osaka, Japan) and sodium hypochlorite (NaClO ; Wako Pure Chemical) were employed. For comparison purposes, a steam high–pressure sterilizer, an autoclave (SS–325, Tomy, Tokyo, Japan) was also employed.

Experimental equipments and procedures :

The equipment employed in this experiment is shown in Fig. 1. A round–bottomed glass vessel with a volume of 200 ml was used as a photocatalytic reactor. The reactor with a magnetic stirring bar was settled on a magnetic stirrer, and the TiO_2 thin film (photocatalyst) was carefully set in the reactor. The sterile distilled water, a volume of 99.9 ml, and a 0.1 ml of the bacterial solution containing 10^6 CFU/ml of *L. pneumophila* were put into the reactor, and then were irradiated with either a 6 W black light lamp (UV–A ; wavelength, 365 nm) or a 6 W germicidal lamp (UV–C;254 nm) in a dark room at 32°C for at least 12 h. A light intensity on the surface of the thin film was nearly 1.00 mW/cm^2, and the aqueous solution was continuously stirred by a magnetic bar at the speed of 100 rpm. Next, 0.1 ml of the water sample was collected, at intervals, to estimate the presence of viable bacteria ; the sample was spread over a BCEY agar plate and then was cultured at 37°C for 3 days to count the colonies of viable bacteria.

The experiments were repeated three times with different cultures of *L. pneumophila*, and similar results were thus obtained each time. The figures demonstrate data from representative experiments. No statistical analysis was performed.

Detection of residual endotoxin :

Two–milliliter of the experimental water solution was sampled at time intervals. After centrifuging 4,000 rpm, the supernatant was collected and used as samples for the detection of any residual endotoxin. To estimate the presence of endotoxin, the samples were diluted 1: 10 in sterile distilled water and incubated for five minutes at 70°C. The concentration of endotoxin was then measured by the QCL–1000 Limulus Amoebocyte Lysate assay (BioWhitaker, Walkersville, Maryland, USA : lower limit of detection 1 ng/ml) using a standard curve created by known concentrations

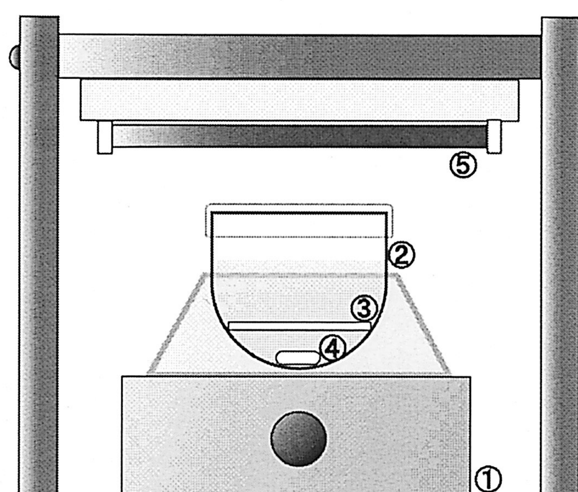

Fig. 1. Equipments employed in the experiment.
① Magnetic stirrer, ② Reactor, ③ TiO_2 thin film photocatalyst, ④ Magnetic bar, ⑤ 6 W black light lamp (UV – A ; wavelength, 365 nm) or 6 W germicidal lamp (UV – C ; 254 nm)

of endotoxin by *Escherichia coli* serotype O111: B4. All determinations were performed in duplicate and the mean of two observations was applied.

Electron microscopy analysis:

For one of the procedures to analyze the killing mechanism of photocatalyst, Scanning electron micrograph (SEM) analysis was carried out with *L. pneumophila* after various treatments. The samples were fixed with 2.5% glutaraldehyde for 2 h at 4℃, and then they were rinsed with 0.1 M NaH_2PO_4 whose pH value was adjusted to 3.0, 7.0 or 11.0 with 1 N HCl and 1 N NaOH. After rinsing, the samples were immersed in a 1% OsO_4 for at least 12 h at 4℃. After being washed with PBS, the samples were dehydrated in a series of aqueous ethanol solutions (70 to 100%), and then were dried with *t*-butanol using a freeze drier (ES-2030, Hitachi, Tokyo, Japan). After iron sputter coating with platinum-paladium of 1.0-1.5 nm, the samples were observed in a high resolution SEM (S-900, Hitachi) at an accelerating voltage of 15-20 kv.

Results

Bactericidal effect of titania thin film photocatalyst under UV irradiation:

In order to investigate the special features of the bactericidal effect of a titania (TiO_2) thin film photocatalyst against *L. pneumophila*, the bacterial water solution in the experimental reactor equipped with or without the photocatalyst was irradiated with a black light lamp (UV-A; wavelength, 365 nm) or a germicidal lamp (UV-C; 254 nm). The survival rates of *L. pneumophila* were determined by counting the number of viable cells in the term of CFU, while comparing the obtained number of cells with the initial numbers of *L. pneumophila* cells (10^3 CFU/ml). As shown in Fig. 2, both UV irradiations, *per se*, yielded a good bactericidal activity, however, in the presence of the TiO_2 photocatalyst, a stronger bactericidal effect was observed with both types of UV radiation. In particular, UV-C irradiation yielded a much stronger bactericidal effect than UV-A; namely, the viable bacteria became undetectable on the TiO_2 photocatalyst within 15 min under UV-C irradiation (Fig. 2b), whereas it took several hours under UV-A irradiation

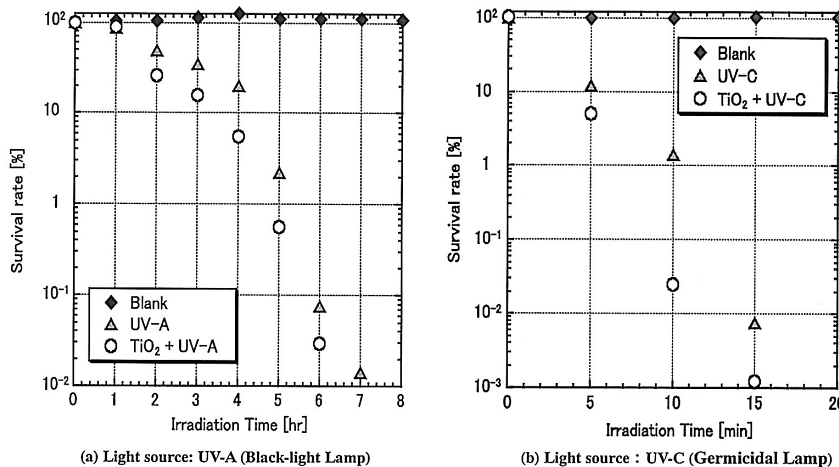

Fig. 2. Time course of survival rate of *L. pneumophila*. 10^6 CFU/ml of *L. pneumophila* on TiO_2 photocatalyst in water were irradiated with either a 6 W black light lamp (UV-A; wavelength, 365 nm) or a 6 W germicidal lamp (UV-C; 254 nm) in a dark room at 32℃ for at least 12 h. Samples were estimated at time intervals for viable cells. Either a UV-A or a UV-C irradiation without TiO_2 thin film photocatalyst and no UV irradiation (blank) were taken as controls.

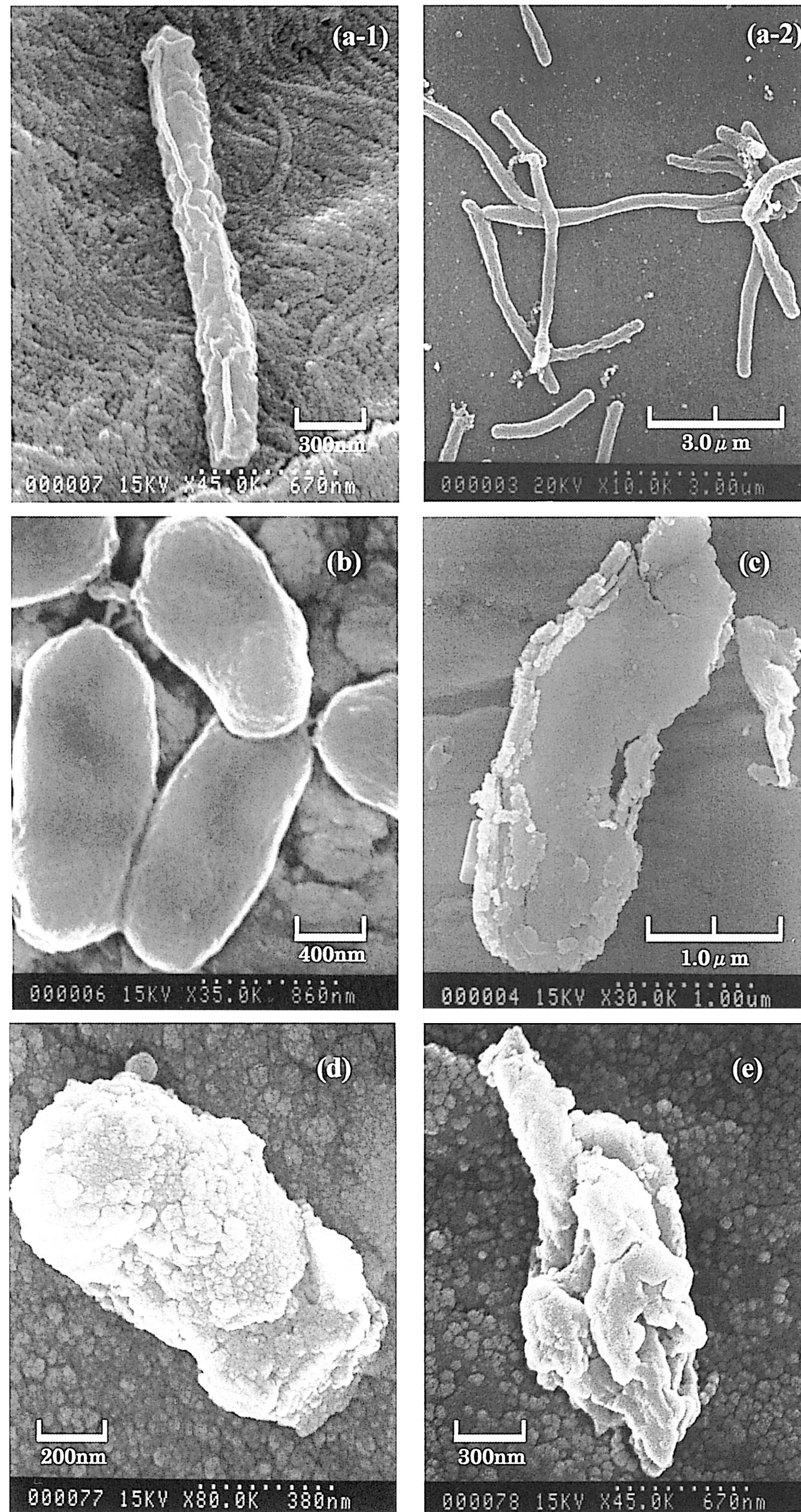

Fig. 3. Scanning electron micrograph (SEM) images of *L. pneumophila* (a–1, 2) incubated for 20 h without UV irradiation (blank), (b) after 20 h–irradiation with UV–A, (c) after 20 h–irradiation with UV–A on TiO_2 photocatalyst, (d) after 20 h–irradiation with UV–C, and (e) after 20 h–irradiation with UV–C on TiO_2 photocatalyst.

(Fig. 2a).

Direct observation of bactericidal effect by SEM :

Fig. 3 shows the electron SEM images of the typical bacteria observed in this study. In general, the genus *Legionella* are slender, pleiomorphic rod bacteria measuring 0.3–0.9×2.0 mm. In length, however, the bacteria vary from short–forms to longer filamentous forms, as shown in Fig. 3a–1 & 2, which represent the bacteria of the non–irradiated control ; it can be seen that no damage has occurred to the cell structure. Fig. 3b shows the bacteria irradiated by UV–A for 20 h without TiO_2 photocatalyst ; it seems that the normal ruffles of structure of their cell surfaces had become rigid, although no destruction of cells was observed. However, either severe damage or a disruption of the cell wall was observed in the bacteria irradiated by UV–A for 20 h in the presence of TiO_2 photocatalyst (Fig. 3c). The UV–C irradiated bacteria showed either a very rough surface or a disruption of the cell wall, even when done without photocatalyst (Fig. 3d). Moreover, when irradiated on the photocatalyst, a severe disruption and dissolution of the bacteria occurred (Fig. 3e). These micrographs support the idea that cell death by the TiO_2 photocatalyst occurs due to a disruption of the cell wall, cell membrane and leakage of the cell contents.

Detoxification of endotoxin by TiO_2 photocatalyst :

As demonstrated in the SEM images in Fig. 3, the UV irradiation of *L. pneumophila* on the photocatalyst caused a severe cell disruption, thus leading to the leakage of cell contents, especially, endotoxin which is the lipopolysaccharide (LPS) cell wall constituent of Gram–negative bacteria. The UV–A irradiation alone did not cause a large amount of endotoxin leakage, and it could not degrade the residual endotoxin during 24 h–irradiation (Fig. 4a ; final concentration, 2,160 pg/ml). However, when the UV–A irradiation was done in the presence of the TiO_2 photocatalyst, nearly 8,000 pg/ml of endotoxin were detected during the first 1 to 3 h–irradiation, but they thereafter gradually degraded during the course of the experiment, and then finally remained undetected after 24 h–irradiation (Fig. 4a). On the other hand, the UV–C irradiation, with or without TiO_2 photocatalyst, caused a much stronger leakage of endotoxin; the highest peak of the amount of leaking endotoxin was observed within 20 min from the start of irradiation in both samples (Fig. 4b). Under UV–C irradiation alone, a half of the endotoxin degraded within 120 min (2 h), but the degradation occurred gradually thereafter until the end of observation ; 1,220 pg/ml of endotoxin was still detected in the last sample (Fig. 4b). However, the UV–C irradiation with the TiO_2 photocatalyst

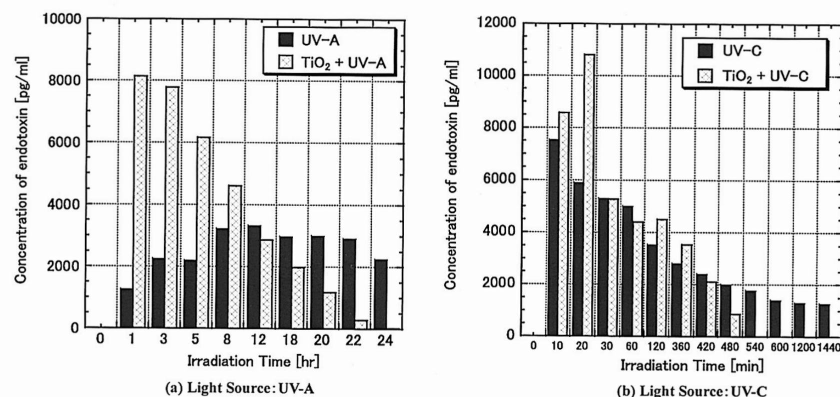

Fig. 4. Time course of the concentrations of endotoxin. During the course of the experiments, samples were collected at intervals for detecting residual endotoxin exhausted from dead bacteria. The estimation of the endotoxin was done by Limulus Amoebocyte Lysate (LAL) assay (see Materials and Methods).

demonstrated a dramatic degradation of endotoxin, which had become undetectable in 540 min (9 h) (Fig. 4b).

Comparison with other disinfectants:

For a better understanding of the disinfectant effect of the TiO_2 photocatalyst, we tested its effect against *L. pneumophila* of other, generally used disinfectants. Approximately 3×10^3 CFUs/ml of cells were treated either with 6 wt% H_2O_2 or 10 ppm NaClO for 24 h. Or, the same numbers of cells were treated with an autoclave at 121℃ for 15 min. Fig. 5 shows the SEM images of *L. pneumophila* after the various treatments. In comparison to the control non-treated bacterium whose cell surface showed rather fine and smooth ruffles (Fig. 5a), all of the three treated bacteria showed very rough or rugged surfaces (Fig. 5b, c, d), although no obvious disruptions of the cell walls were observed in these cells. Nearly 10 CFU/ml of viable *L. pneumophila* were detected after the 24 h-treatment with H_2O_2, whereas no viable bacteria were detected after the NaClO treatment. No leakage of endotoxin was noticeable with the treatment of

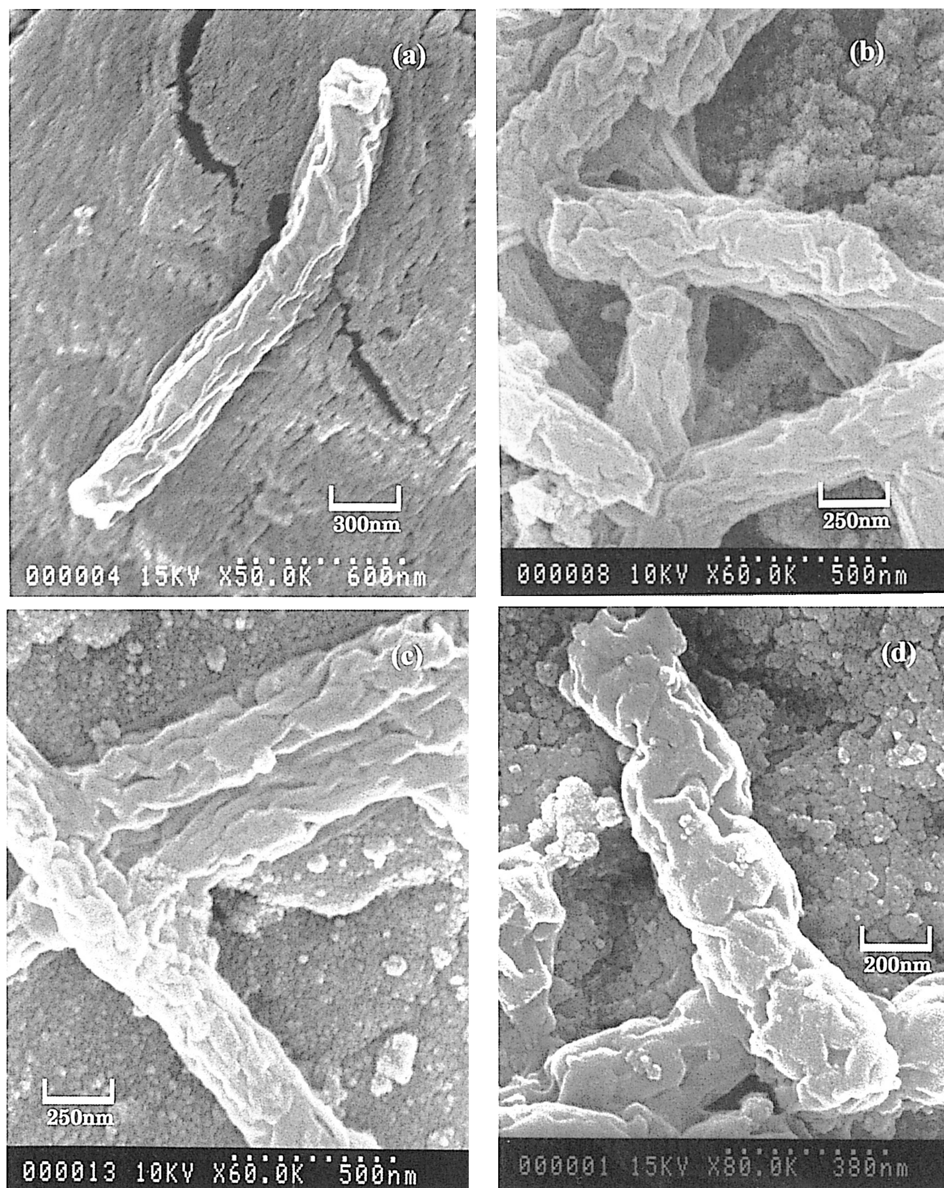

Fig.5. Scanning electron micrograph (SEM) images of *L. pneumophila* treated with (a) no disinfectant (control), (b) 6 wt % H_2O_2 for 24 h, (c) 10 ppm NaClO for 24 h, and (d) heat (121℃) for 15 min.

these disinfectants (up to 2,000 pg/ml), but 30 to 85% of residual endotoxin were still detected even after the 24 h-treatments in the both treated samples. Even after thermal treatment of the bacteria with an autoclave, 35% of the estimated amount of endotoxin (exhausted from 3×10^3 CFUs/ml of cells) were detected.

Discussion

Recently, increasing attention has been given to the photocatalyst to destroy organic compounds or microorganisms in contaminated air and water, and titania dioxide (TiO_2) is the most widely used photocatalyst. Upon excitation by light whose wavelength is less than 385 nm, the photon energy generates an electron hole pair on the TiO_2 surface.[10] The hole in the valence band can react with either H_2O or hydroxide ions absorbed on the surface to produce hydroxyl radicals (OH●), and the electron in the conduction band can then reduce O_2 to produce superoxide anion (O_2^-). The detection of other reactive oxygen species (ROS), such as hydrogen peroxide and singlet oxygen, has also been reported.[16][22] These ROS generated by the TiO_2 photocatalyst are extremely reactive with contacting organic compounds.[27]

Based this feature of TiO_2 photocatalysis, many studies have been done on its bactericidal activity against microorganisms; the inactivating effects on *Escherichia coli*,[13][17][20] *Streptococcus mutans*[19] and *Pseudomonas aeruginosa*[14] have been investigated. In the present study, we first examined the bactericidal activity against *L. pneumophila* of a TiO_2 thin film photocatalyst that was prepared in our laboratory.

The *Legionella* species are environmentally abundant bacteria which cause severe pulmonary syndromes (legionellosis) in humans such as Legionnaires' disease. The legionellosis is acquired from environmental sources, such as cooling towers, whirlpool spas (hot-springs) and even potable water systems.[28] In July 2002, a large outbreak of legionellosis occurred in a bathhouse with spa facilities in Miyazaki Prefecture, in Japan.[9] Two hundred-ninety-five patients (including suspected cases) who had pneumonia and/or symptoms of fever, cough and so forth were reported; 37% of them were hospitalized and seven people died. For the transmission of *Legionella* species to humans, colonization of these water sources by the bacteria is necessary, and preventive steps have thus been taking to cope with this bacterial colonization. Chemical disinfectants including chlorinated compounds, such as chlorite and monochloroamine, have been widely used to prevent the bacterial colonization of water sources,[28][29] but the overuse of these chemical compounds also causes a risk of environmental pollution as already stated before. Therefore, if the TiO_2 photocatalytic process can prove to be sufficiently effective for inactivating bacteria, then it might be a promising new technology for sterilizing water sources. Our results showed that a TiO_2 thin film prepared in our laboratory exerted, together with UV-irradiations, a strong and effective photocatalytic bacterial killing against *L. pneumophila* (Fig. 2a and b). These results are basically consistent with the findings of Matsunaga et al.,[11][17] Saito et al.[19] and Sunada et al.[13] that were done on *E. coli* or *Streptococci*. However, in their works, powdered TiO_2 preparations were used as photocatalysts, or, bacterial samples were directly placed on the TiO_2 plate in order to obtain closer contact to a photocatalyst. In our study, we set up an experimental condition as a model approaching a future practical use (Fig. 1); i.e., our study was done using a TiO_2 thin film in a fully aqueous condition. Moreover, in the present study, observation by SEM showed that the TiO_2 photocatalyst with UV-irradiations caused a disruption and/or even a dissolution of the cells walls (Fig. 3c and e), as reported by Amezaga-Madrid et al.[14] who did an experiment using *Pseudomonas aeruginosa* placed on the TiO_2 photocatalyst. The destruction of the cell walls promoted the leakage of endotoxin from the dead bacteria (Fig. 4). UV-irradiation alone and other disinfectants such as H_2O_2, NaClO and heat treatment (121℃) induced severe conformation changes in the bacterial cell walls possibly due to the denaturation of their integral components (Figs. 3b, 3d and 5b, c, and d), but no obvious destruction of cell walls was observed.

The most prominent finding of our results is that the TiO_2 thin film photocatalyst was able to completely degrade or detoxify endotoxin within a

short time when the photocatalyst was exposed to the UV irradiations (Fig. 4). Endotoxin, an integral component of the outer membrane of Gram–negative bacteria, is exhausted from dead bacteria. The problem is that the endotoxic activities such as pyrogenicity and lethal toxicity are elicited even at very low concentrations. It is therefore extremely important to eliminate or deactivate endotoxin, as well as to kill the bacteria, themselves. In general, endotoxin in aqueous solution cannot be easily deactivated, usually requiring membrane filtration, adsorption by activated carbon, or affinity adsorption by polymyxin.[30] In the samples used in this study, treated with either H_2O_2 or NaClO, fairly large amounts of residual endotoxin were still detected even after the 24 h–treatments, although DeRenzis[31] reported that H_2O_2 decomposed the lipid–A of endotoxin by oxidation. Because Hashimoto and Fujishima[32] showed that the oxidizing activities of H_2O_2 and chlorinated compounds were much weaker than those given off by UV–irradiated TiO_2 film, our results may thus confirm the findings of their report. Moreover, after a thermal treatment at 121℃ for 15 min with an autoclave, 35% of the estimated amount of exhausted endotoxin remained undegraded, which confirmed the previous finding of Bamba et al.[33] that it required 250℃ for more than 30 min to complete the deactivation of endotoxin. On the other hand, the UV–irradiated TiO_2 photocatalyst could effectively degrade endotoxin in an aqueous solution (Fig. 4) due to its strong oxidizing power.[13] The UV–irradiation, *per se*, without TiO_2 photocatalyst could also degrade endotoxin, but the efficacy of their degradation activities seemed to be insufficient (Fig. 4). The concentration of residual endotoxin in the test solution was quite high in the first 20 min or 1 to 5 h with UV–C or UV–A irradiation on the photocatalyst (Fig. 4). The increased concentration of the endotoxin means that the TiO_2 photocatalyst destroys the outer membrane of *L. pneumophila* cells (Fig. 3c and e). The difference in the degrading efficacy between UV–A and UV–C is possibly due to the difference of oxidizing energy produced from the TiO_2 thin film by UV–irradiations.[34]

We herein examined the photoinduced activities of the TiO_2 photocatalyst under UV–irradiations, UV–A (black–light ; wavelength, 365 nm) and UV–C(germicidal–light;254 nm). No effective bactericidal activity was elicited by the photocatalyst with the use of white–light lamp for general lighting (data not shown). Although UV–C irradiation showed a much stronger effect than UV–A irradiation, in this study we confirmed that the UV–A ray, a safer one for living organisms than UV–C ray, was also effective in photoinduced excitation of a TiO_2 film.

In conclusion, our results presented herein show that the UV–irradiated TiO_2 photocatalyst has both bactericidal activity and a degrading or detoxifying activity for endotoxin, due to both the inactivation of the viability of *L. pneumophila* and the disruption of the bacteria. These features suggest that the TiO_2 photocatalyst may thus be a potentially effective means for carrying out environmental protection, not only for cooling towers, public baths or spas, and potable water systems, but also for medical facilities where endotoxins also need to be controlled.

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