Note

The Effects of Peptidoglycan on the Photocatalytic Bactericidal Activity of Titanium Dioxide

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For the elucidation of the mechanism underlying the photocatalytic bactericidal activity of titanium dioxide (TiO₂), we focused on the peptidoglycan layer, a component of the bacterial cell wall. The effect of this layer on the photocatalytic bactericidal activity of TiO₂ was evaluated by determining the survival rates of *Lactobacillus plantarum* (intact cells) and its protoplast cells. *Mesoplasma florum*, which does not originally possess the peptidoglycan layer, was also used. Our results revealed that the survival rates of the intact cells were lower than those of the protoplast cells. In addition, there was no significance between the survival rates of *M. florum* cells and the protoplast cells of *L. plantarum*. It was suggested that the presence of the peptidoglycan layer increases the bactericidal effect by the photocatalysis.

Key words : Photocatalysis / TiO₂ / Bactericidal effect / Protoplast / Peptidoglycan.

Various environmental problems such as water and air pollutions are caused by microorganisms and must be addressed. Antibiotics, antibacterial drugs, disinfectants, and other bactericidal agents have been developed as a solution to these issues; however, problems such as the appearance of drug-resistant microorganisms and high drug development costs still persist. For these reasons, a new sterilization technique for overcoming these difficulties is required. As one such promising technique, photocatalysts are attracting attention. Reactive oxygen species (ROS) are generated on the surface of TiO₂ following the irradiation of UV light. Such ROS can ultimately decompose organic matter to CO₂ and H₂O (Carré et al., 2014; Fujishima and Honda, 1972; Fujishima et al., 2008; Noguchi et al., 1998; Sopyan et al., 1996). However, there is no clear knowledge regarding the mechanism underlying the bactericidal effect of photocatalysis. While gram-positive bacteria have been reported to be less sterile than gram-negative bacteria owing to the presence of a thick peptidoglycan layer (Kühn et al., 2003; Pal et al., 2007), gram-positive

bacteria are reportedly more easily sterilized (Tsai et al., 2010). Another study has reported that ROS only passes through the peptidoglycan layer and the reaction occurs only in the outer and inner membranes (Sunada et al., 2003). As mentioned above, the results of photocatalysis in the cell wall are currently contradictory. Therefore, this study aims to assess the influence of peptidoglycan in the cell wall on the photocatalytic activity of TiO₂ to clarify the mechanism underlying sterilization.

To evaluate the bactericidal effect by the peptidoglycan layer, we used gram-positive bacteria with a thick peptidoglycan layer. A Gram-positive bacterium generally used in research is *Bacillus subtilis*. However, *B. subtilis* is a spore-forming bacterium. We thought that the effect of the photocatalysis might be different between spores and vegetative cells. Therefore, we used *Lactobacillus plantarum*, which does not form spores and has a thick peptidoglycan layer. *L. plantarum* intact cells with a very thick peptidoglycan layer and *L. plantarum* protoplast cells without a peptidoglycan layer were used to confirm whether the bactericidal effect differs depending on the presence or absence of peptidoglycan layer as the main component of the cell wall. Since *L.*

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plantarum is a rod-shaped bacterium, it can be readily distinguished from protoplast cells rounded by removing the cell wall.

L. plantarum JCM1149^T was used for the bactericidal experiments. This strain was obtained from the Japan Collection of Microorganisms. *L. plantarum* JCM1149^T cells were statically cultured in de Man, Rogosa, and Sharpe (MRS) broth (Difco) at 30 °C for 3.5 h until early log phase. The cells were washed twice with 0.2 M phosphate-buffer (pH 6.8) and once with MASH buffer (pH 6.5) contained (per liter): 4.8 g of HEPES, 6.0 g of MgCl₂·6H₂O, 684.6 g of sucrose and 5.0 g of gelatin. The composition of MASH buffer was determined by consideration of conditions to be optimal in maintaining the form of protoplast cells. The cells were re-suspended in MASH buffer to OD (Optical Density) at 660 nm of 0.5. This solution was used as the bacterial suspension of intact cells. The bacterial suspension of protoplast cells was prepared as follows. First, Lysozyme (from Egg White, Wako Pure Chemical Industries, Ltd.) and Labiase (Cosmo Bio Co., Ltd.) were mixed in MASH buffer at 15 mg/mL each to prepare the enzyme solution. This enzyme solution (180 µL) was added to the intact cells suspension (1 mL) and treated at 37 °C for 30 min. This solution was used as the bacterial suspension of protoplast cells.

We investigated the bactericidal effect of photocatalyst using TiO₂ coated glass, which does not need into consideration in nanoparticle toxicity. The preparation of TiO₂ coated glass was performed as follows. Borosilicate glass (TEMPAX: 50 × 50 × 2 mm, Hiraoka Special Glass Mfg. co., Ltd.) was washed with acetone, ethanol and water, and ozone treated by UV Ozone Cleaner UV253E (Filgen, Inc.) for 30 min. The ozonized glass was spin-coated (1,500 rpm, 10 s) with 1 mL of NDH-510C (TiO₂, Nippon Soda Co., Ltd.) by Spin coater (MS-A200, Mikasa Co., Ltd.). This TiO₂ coated glass was baked at 500 °C for 2 h in a muffle furnace (KDF-S70, Sansyo Co., Ltd.).

The photocatalytic effect on bacteria was evaluated to assemble the equipment based on Japanese Industrial Standards (JIS R-1702). All the constituent parts of the equipment were washed with 70% ethanol and irradiated with UVC overnight. The TiO₂ coating glass was placed on a plastic stage on a sheet of filter paper moistened in sterile water placed in a petri dish. 0.3 µL of LIVE/DEAD[®] BacLight[™] Bacterial Viability Kit solution (Molecular Probes) was added each to 100 µL of the bacterial suspension of intact cells and protoplast cells. Each 10 µL of the sample was dropped on the different TiO₂ coating glass. 22 × 22 mm cover glass was placed thereon, and the petri dish was covered with a wrap. The irradiation intensity of UVA (FL15BL-B, Panasonic) was adjusted to 0.25 mW/cm² (Fig. 1). This irradiation

intensity is equivalent to the intensity of sunlight that is inserted into the window. Since LIVE/DEAD[®] BacLight[™] Bacterial Viability Kit is discolored by UVA irradiation, the photocatalysis was performed for up to 2 h. After the photocatalysis, the survival rate was calculated from the numbers of green fluorescent cells as live cells and red fluorescent cells as dead cells using a confocal laser microscope (LSM EXCITER-ZEN 2008, ZEISS). The conventional survival rate evaluation is based on the colony count method. In this method, the operation of spreading the bacterial solution on an agar plate is required. However, *L. plantarum* protoplast cells are weak to physical stress due to their lack of cell walls. Therefore, the survival rate evaluation by LIVE/DEAD[®] BacLight[™] Bacterial Viability Kit, which is a viability test based on the degree of membrane damage, was conducted instead of the colony count method.

A photocatalysis was performed on the protoplast cells and the intact cells of *L. plantarum*. The survival rate of the protoplast cells decreased to 86% for 1 h of photocatalytic treatment and to 85% for 2 h. The survival rate of the intact cells decreased to 64% for 1 h of photocatalytic treatment and to 50% for 2 h. Statistical analysis (Student's t-test) revealed that the time course of survival rates by photocatalytic treatment was significantly different between the protoplast cells and the intact cells (Fig. 2). The result that the survival rate of the intact cells was significantly lower than that of the protoplast cells led to the idea that the reduction of the survival rate was suppressed by the protein added to prepare the protoplast cells.

In order to ascertain the influence of cell wall digesting enzyme added at preparing protoplast cells, the survival rate was evaluated by adding albumin without Lysozyme and Labiase activity. After the addition of albumin to the suspension of the intact cells, the photocatalytic treatment was performed in the experimental equipment (Fig.1). The final concentration of the albumin was the same as the optimum cell wall digesting enzyme concentration for the preparation condition of the protoplast cells. The survival rate of the intact cells without albumin decreased to 91% for 1 h of the photocatalytic treatment and to 75% for 2 h. The survival rate of the intact cells with albumin decreased to 98% for 1 h of photocatalytic treatment and to 79% for 2 h. According to the results of statistical analysis, there were no significant differences in the effects of the photocatalytic reaction of the intact cells with or without protein (Fig 3). Therefore, the possibility that the decrease in the survival rate was suppressed by the presence or absence of the addition of the protein was denied. Also, the survival rate of the protoplast cells was 99% even for 2 h of photocatalytic treatment. According to the results of statistical analysis, there was a significant difference

FIG. 1. The experimental equipment using in photocatalytic treatment.

- (A) The experimental equipment was based on JIS R-1702. Photocatalytic treatment was carried out in this experimental equipment. A sheet of filter paper containing 4 mL of sterilized water was laid on the bottom so that the bacterial solution was not dried by UVA irradiation heat.
- (B) Inside the UVA irradiation device for photocatalytic treatment. Three UVA lamps are installed at the top inside the device. The plastic Petri dishes were stacked to make a stage so that the UVA intensity of each top of the stage was 0.25 mW/cm². "A" was placed on the stage, and the photocatalytic treatment was performed.

in the photocatalytic reaction effects between the protoplast cells and the intact cells added protein (Fig 3). In other words, the difference in the survival rate between the protoplast cells and the intact cells for 2 h of the photocatalyst treatment in Fig. 3 is considered to be due to the presence or absence of the peptidoglycan layer.

L. plantarum protoplast cells are the cells from which the peptidoglycan layer has been removed by enzymatic treatment. That is, it is difficult to assert that the protoplast cells have completely lost the peptidoglycan layer. Therefore, in order to strictly evaluate the effect of the peptidoglycan layer, we also used *Mesoplasma florum* NBRC100688^T which originally have no cell wall. This strain was obtained from the National Institute of Technology and Evaluation. *M. florum* cells were cultured in ATCC 1161 medium at 30°C with shaking for 18 h until early log phase. The cells were washed with Serum-Sucrose-HEPES (RUSH) buffer (pH 6.5) contained (per liter): 0.6 g of HEPES, 22.6 g of NaCl, 0.39 g of 2-Mercaptoethanol, 0.25 g of sucrose and 250 mL of

Horse serum. The cells were re-suspended in RASH buffer to OD (Optical Density) 660 nm of 0.5. This solution was used as the bacterial suspension of *M. florum* cells. The survival rate of *M. florum* cells decreased to 98% for 2 h of the photocatalytic treatment. Also, the survival rate of *L. plantarum* protoplast cells decreased to 99% for 2 h of the photocatalytic treatment. Statistical analysis showed that there was no significant difference between the photocatalytic bactericidal susceptibilities of *M. florum* cells and the protoplast cells of *L. plantarum* (Fig 4). Therefore, it showed that the survival rate of the cells without the peptidoglycan layer did not decrease in the photocatalysis for 2 h.

To summarize above, it was revealed that the survival rate of the cells possessing the peptidoglycan layer tends to decrease, and the survival rate of the cells without the peptidoglycan layer is unlikely to decrease. That is, it is strongly suggested that the peptidoglycan layer might enhance the bactericidal effect by photocatalysis.

FIG. 2. The survival rates of the protoplast cells and the intact cells by the photocatalytic treatment.

The survival rate of the protoplast cells was about 86% for 1 h, about 85% for 2 h of the photocatalytic treatment. There was no significant difference from the survival rate of the negative controls. The survival rate of the intact cells was about 64% for 1 h, about 50% for 2 h of the photocatalytic treatment. There was significant differences from the survival rate of the negative controls ($[\text{TiO}_2 (+), \text{UVA} (-)]: p < 0.05$, $[\text{TiO}_2 (-), \text{UVA} (+)]: p < 0.001$). Comparing the survival rate of the protoplast cells and the intact cells by the photocatalytic treatment, there was a significant difference in the viability of both cells ($p < 0.001$). "n" indicates the number of times of the photocatalytic treatment experiment was performed.

FIG. 3. The survival rate of the intact cells with added albumin by the photocatalytic treatment.

The survival rate of the intact cells without albumin addition was about 91% for 1 h, about 75% for 2 h. On the other hand, the survival rate of the intact cells with albumin addition was about 98% for 1 h, about 79% for 2 h. There were no significant differences between these conditions ($p < 0.05$). The survival rate of the protoplast cells was about 99% even for 2 h. The significant difference was observed between the intact cells with albumin addition and the protoplast cells ($p < 0.01$). "n" indicates the number of times of the photocatalytic treatment experiment was performed.

FIG. 4. The comparison between the survival rates of *M. florum* cells and the protoplast cells by the photocatalytic treatment.

The survival rate of *M. florum* cells originally without cell wall was about 98% even for 2 h. There was no significant difference between the survival rates of *M. florum* cells and the protoplast cells ($p < 0.05$). "n" indicates the number of times of the photocatalytic treatment experiment was performed.

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