Direct observation and analysis of bacterial growth on an antimicrobial surface

Running title: Micro-culture of bacteria on an antimicrobial surface

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Abstract

Cells of *Escherichia coli* NBRC 3972 and *Staphylococcus aureus* NBRC 12732 were inoculated on an agar (1.5%) medium varying in nutrient concentration from full strength of the nutrient broth (NB) to 1/10 NB. Immediately thereafter, the inoculated agar was placed on antimicrobial and non-antimicrobial surfaces in such a way that the microbial cells came into contact with these surfaces. Cell growth was directly observed under a microscope, and the growth rate constant of the cells was measured based on the increase in the area of the colonies formed by the growing cells. On the antimicrobial surface, the growth rate constant decreased at lower nutrient concentrations for both *E. coli* and *S. aureus* cells, whereas it showed little change on the non-antimicrobial surface. It was supposed that either the nutrient uptake or the nutrient utilization efficiency was retarded by the antimicrobial surface. At the lowest nutrient concentration examined in the present study, 1/10 NB, the cells could hardly grow on the antimicrobial surface, indicating that the surface would be sufficiently active in preventing bacterial growth under normal usage conditions, such as the wet areas of a kitchen. It was also revealed that the antimicrobial surface could prevent the
division of cells either during the growth stage or before the onset of growth.

Introduction

Antimicrobial materials have been used in various contexts worldwide, such as dental and medical devices (13, 21), chronic-wound dressing (10), biofilms (11), stainless-steel surfaces (e.g., air ducts, countertops and food preparation areas) (7) and frequently hand-touched surfaces (2). However, concerns associated with the overuse of antimicrobial materials and the consequent emergence of bacterial resistance are being raised (15). It has been shown that the production of extracellular polymeric substances (slime) decreases the susceptibility of microbes to materials loaded with antibiotics (4, 14). The resistance can be mediated by efflux of plasmids carrying genes for antimicrobial agents (17). The range of bacterial response to antimicrobial materials is extensive. So, the precise evaluation of the antimicrobial activity of various antimicrobial materials is important for their further application and to prevent problems caused by the improper utilization of these materials.

The activity of antimicrobial materials is conventionally estimated using an
indirect method in accordance with ISO 22196 (JIS Z2801; 2000) (24). In this method, a cell suspension of *Escherichia coli* NBRC 3972 or *Staphylococcus aureus* NBRC 12732 is inoculated onto a surface, and then the cells are collected from the surface and examined as to whether or not they can form colonies on nutrient agar. The ratio of the number of cells able to form visible colonies to the number of cells contacting the surface is considered to be an index of the antimicrobial activity of the surface. However, the activity of the cells that are in contact with the antimicrobial surface has yet to be elucidated.

Antimicrobial materials have been used in various environments such as kitchens, rest rooms and bathrooms, where the concentration of nutrients available to domestic microorganisms varies. It has been reported that the antimicrobial activity of a material differed depending on the nutrient concentration (23). In the present study, to examine more precisely the antimicrobial activity at different nutrient concentrations, the cell growth of *Escherichia coli* NBRC 3972 and *Staphylococcus aureus* NBRC 12732 on the surfaces of non-antimicrobial and antimicrobial materials was investigated at various nutrient concentrations.
In the present study, the effect of an antimicrobial material was examined by directly observing the cells growing on its surface through a microscope. By this method, the antibiotic activity can be evaluated quantitatively and more precisely. The direct observation of growing cells also led to some interesting findings which will deepen the understanding of the hitherto controversial mechanism behind the antimicrobial activity of certain materials.

Materials and Methods

Culture and preparation of bacterial cells

In this study, the strains prescribed in ISO 22196 for the evaluation of antimicrobial activity were used. The strains *Escherichia coli* NBRC 3972 (isolated from human feces (8)) and *Staphylococcus aureus* NBRC 12732 (isolated from the abscesses of young sheep (6)) were obtained from the Biological Resource Center of the National Institute of Technology and Evaluation in Japan. The cells were cultured in NB or diluted NB (1/2, 3/8, 1/4, 1/10) liquid medium at 27 °C with shaking (100 revolutions per min) until the stationary phase (approximately 24 h). The cultured cells were diluted
with distilled water to a concentration of $10^8$ cells/mL, and the cell suspensions were used for the micro-culture.

Micro-culture of bacterial cells on non-antimicrobial and antimicrobial surfaces

Bacterial growth was directly observed under a microscope (BX50, Olympus, Tokyo, Japan) and photographed, as shown in Figure 1. A cell suspension of about 4 microliters ($10^8$ cells/mL) was inoculated on the surface of a small block of nutrient agar (1.5%; 14 x 14 x 0.3 mm) by spreading it over the surface with an inoculating needle. Immediately thereafter, the agar was placed gently and face down on an antimicrobial material (GPPS1.0%; high-impact polystyrene plate with 1.0 % of an antimicrobial agent named Novaron AG300 (Toagosei, Tokyo, Japan)) or a non-antimicrobial material (GPPS BL; the same material as the antimicrobial one without the antimicrobial agent) used as a control. Then, a cover glass (18 x 18, thickness 0.12 - 0.17 mm, Matsunami, Tokyo, Japan) was placed over the material. The margins of the prepared specimens were sealed with a sealant to prevent desiccation. The specimens were kept on the stage of the microscope during the experiment at an
almost constant temperature around the microscope of 26°C.

When the microbial cells grew between the solid (non-antimicrobial or antimicrobial material) surface and the nutrient agar, they spread horizontally with time as new cells grew next to existing ones (Fig. 2). Micro-scale colonies consisting of a number of cells were observed through the microscope and photographed intermittently. As the cells grew, the total area of the micro-scale colonies increased with time, because of the increase in the number of cells comprising the colonies. Thus, from the increase in the colony area, the growth rate of the cells could be estimated.

Calculation of the growth rate constant

We observed and photographed the cells growing under the nutrient agar directly with the microscope in 2 - 4 fields (width of one field: 160 x 220 µm) at each observation occasion. The same fields, secured with a guide gage mounted on the microscope, were used to follow the growth of each colony. The photographs of the fields were printed out and the portions of the image showing the colonies were darkened with a thin wash of black ink to enhance the signal/noise ratio. Finally, the
colony area was calculated by means of an image analyzer (Scion Image, Scion Corporation, MD, USA): 1 \( \mu \text{m} \) corresponded to 5 pixels in the present study. The colony area increased proportionally to the cell number as the cells grew, so the colony area could be used to evaluate the cell growth. Furthermore, the colony area increased not at intervals like the cell number, but continuously as each cell grew larger from one cell division to the next. Thus, the change in the colony area seems to give us a more precise growth rate constant than that obtained by using the cell number. Therefore, we adopted the colony area to describe the cell growth, as follows:

\[ S = S_0 \exp(\mu t) \quad , \quad (1) \]

where \( S \) and \( S_0 \) are the colony areas at times \( t \) and \( t_0 \), respectively.

By taking the natural logarithms of both sides of the above equation, we obtain the following equation:

\[ \ln S = \ln S_0 + \mu t \quad . \quad (2) \]

Thus, by plotting the natural logarithm of the colony area against the incubation time, we can obtain the growth curve of the cells on the surface, and the slope of a part of the curve gives the growth rate constant of the cells. The average size of the cells
composing the colony was determined by dividing the colony area by the number of colony-bound cells which could be counted from the image.

Results and Discussion

Bacterial growth on the non-antimicrobial and antimicrobial surfaces

Antimicrobial materials have been used in various environments such as kitchens, rest rooms and bathrooms, where the concentration of nutrients available to domestic microorganisms varies. It has been reported that the antimicrobial activity of a material differed depending on the nutrient concentration (23). To study the antimicrobial activity at different nutrient concentrations, the cell growth of *Escherichia coli* NBRC 3972 and *Staphylococcus aureus* NBRC 12732 on the examined surfaces (non-antimicrobial and antimicrobial) was investigated by varying the concentration of the nutrient.

The bacterial cell growth on the solid surface under the nutrient agar was observed directly under a microscope. Although limited to transparent antimicrobial materials, this method presents a useful and simple way to observe microbial growth on
the antimicrobial material and is applicable under a wide variety of conditions such as varying nutrient concentrations and varying types of cells used. The area of the micro-scale colonies consisting of a number of cells, as well as the natural logarithm of the colony area, increased with the incubation time because of cell growth (data not shown). The slope of the linear relationship between the incubation time and the natural logarithm of the colony area gives the growth rate constant of the cells.

Antimicrobial activity at various nutrient concentrations

The growth rate constant on the non-antimicrobial surface (GPPS BL) differed little under different nutrient concentrations, from full-strength nutrient broth (NB) to 10-fold-diluted nutrient broth (1/10 NB), for both \textit{E. coli} and \textit{S. aureus}, as shown in Figure 3. This means that, even at 1/10 NB, the nutrient concentration was high enough to support the normal growth of these cells; in other words, the cells can take up the nutrient in high enough quantities to allow them to produce new cells over a given time period even at 1/10 NB.

On the antimicrobial surface (GPPS 1.0%), the cells of \textit{E. coli} showed almost
the same growth rate constant as on the non-antimicrobial surface when growing at higher nutrient concentrations, from NB to 1/2 NB (Fig. 3). In contrast, at lower nutrient concentrations (less than 3/8 NB), the growth rate constant decreased on the antimicrobial surface, as shown in Figure 3.

Although the cells of *E. coli* did not grow at 1/4 and 1/10 NB on the antimicrobial surface, at other nutrient concentrations the cell size of *E. coli* was almost the same on both types of surface (Fig. 4). To produce these cells, the same amount of nutrient must be used regardless of the difference in growing conditions, such as the nutrient concentration and the surface with which the cells come in contact. If the nutrient uptake by the cells or the nutrient utilization efficiency is retarded, the cells will need a longer time to produce new cells, resulting in a smaller growth rate constant.

Although it is not clear which of the possible mechanisms mentioned above is actualized, the antimicrobial surface seems to affect the cells so as to decrease the nutrient uptake activity or the nutrient utilization efficiency. This retarding effect was not apparent at higher nutrient concentrations, whereas it became clear at lower nutrient concentrations.
The cells of *S. aureus* as well as those of *E. coli* showed a smaller growth rate constant on the antimicrobial surface at nutrient concentrations lower than 3/8 NB compared with the non-antimicrobial surface (Fig. 3), and, at 1/10 NB, the cells hardly grew. The cell size was almost the same at all nutrient concentrations on both types of surface (Fig. 4). As discussed above for *E. coli*, the uptake of the nutrient or the nutrient utilization efficiency seems to be retarded at lower nutrient concentrations for the cells of *S. aureus* as well, especially at 1/10 NB, on the antimicrobial surface.

The cells of both *E. coli* and *S. aureus* could hardly grow on the antimicrobial surface at 1/10 NB. The 1/10 NB nutrient contains about 43 micrograms sugar as the glucose equivalent and about 9 micrograms protein as the BSA equivalent per milliliter (mL). These values are much higher compared with those of tap water (below 5 micrograms/mL for both sugar and protein). So, the antimicrobial surface used in this study (GPPS 1.0%) should be sufficiently effective in preventing bacterial growth in wet areas of the household.

**Mechanism causing antimicrobial activity**
Several hypotheses have been put forth regarding the mechanism behind antimicrobial activity. Silver ions are known to have an antimicrobial effect: they strongly interact with the thiol groups of enzymes and inactivate them (9, 12). Silver ions are also known to inhibit oxidative enzymes such as yeast alcohol dehydrogenase (18). Other studies have uncovered evidence that silver ions inhibit the uptake of phosphate (16) and disturb the respiratory chain of *Escherichia coli*, cause the efflux of metabolites (1), and are related to the formation of free radicals and subsequent free radical–induced membrane damage (3). Another mechanism is the formation of “pits” in the cell wall of Gram-negative bacteria, depending on the concentration of the ambient silver nanoparticles (19, 20).

The antimicrobial material used in the present study was a mixed and molded product made of an inorganic antimicrobial agent (Novaron AG300) composed of silver-based ion exchangers in hexagonal phosphate zirconium and an organic material, polystyrene. Novaron is presumed to exert its antimicrobial effect through silver ions excreted from the material and/or activated oxygen generated from water in the presence of Novaron and light, according to the manufacturer’s account.
In the present study, we kept the micro-culture sample on the stage of the microscope and illuminated the sample for just 1-2 minutes at each observation occasion (less than 30 minutes in total during an experiment lasting for 20-30 hours). Thus, activated oxygen was hardly produced under the experimental conditions of the present study. On the other hand, the rate of release of silver ions from the material, although quite small, is reported to be enhanced in the presence of amino acids and protein (22). Thus, in the presence of nutrient agar, silver ions seem to have been released from the material to a certain extent during the incubation period in the present study, and may have affected the microbial activity through the mechanisms as mentioned above.

Although the results obtained in the present study do not prove directly that silver ions were released from the material, a physiological effect of the antimicrobial surface was revealed in the present study. Namely, it became clear that the nutrient uptake activity or the nutrient utilization efficiency of the bacterial cells was retarded on antimicrobial surface. Additionally, we obtained two kinds of results which suggest that the bacterial cell cycle is disturbed on the antimicrobial surface, as described below.
The cells of *E. coli* and *S. aureus* formed cells colonies under the nutrient agar, as shown in Figure 5. These colonies started from one cell and increased in size with time. On the antimicrobial surface, the increase in colony size stopped earlier; thus, smaller colonies were formed compared with those on the non-antimicrobial surface. This was much more obvious at lower nutrient concentrations, as shown in Figure 5.

The antimicrobial surface seems to interrupt the cell cycle and prevent further cell division. Silver ions may accumulate in the DNA of the microbial cells (5) during the incubation and the DNA may lose its ability to replicate.

The same cell suspension was inoculated onto a nutrient agar surface and spread uniformly over the surface before placing the agar on a non-antimicrobial and an antimicrobial surface in these experiments, so that the cells would be evenly distributed and a similar colony number would result for both surfaces in case the antimicrobial surface had no effect on cell growth. This was the case at NB (full-strength nutrient). Also, a large number of colonies (over 100) were counted per mm\(^2\) for both *E. coli* and *S. aureus* at 1/10 NB on the non-antimicrobial surface. However, no colony was observed on the antimicrobial surface at 1/10 NB for either *E. coli* or *S. aureus*. This
means that the antimicrobial surface blocks the initiation of the cell cycle at the nutrient concentration of 1/10 NB.

In the present study, it became clear that the antimicrobial surface can prevent the cell growth of *E. coli* and *S. aureus* by disturbing the cell division process and by decreasing the growth rate constant through retarding the nutrient uptake or lowering the nutrient utilization efficiency. These findings must be further confirmed by using kinds of bacteria other than *E. coli* and *S. aureus*. Also, the mechanism behind the phenomena observed in the present study must be further analyzed in a future study.

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**References**


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antimicrobial effect of a product processed with an inorganic silver antibacterial. Bokin

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Figure captions

1. Figure 1. Schematic illustration of the micro-culture method used in this study.

2. Figure 2. Process of cell growth of *S. aureus*, as observed by the micro-culture method. The number of cells comprising the colony, and therefore the colony size, increased with the incubation time (A: 80 min; B: 740 min; C: 1,212 min).

3. Figure 3. Growth rate constants of *E. coli* (A) and *S. aureus* (B) on the antimicrobial (GPPS 1.0%) and non-antimicrobial (GPPS BL) surfaces at various nutrient concentrations. The experiments were repeated two or three times, independently. From the growth rate constants obtained for ca. 10-20 colonies, the mean and standard error (shown as dots and error bars, respectively) were calculated.

4. Figure 4. Cell sizes (μm²) of *E. coli* (A) and *S. aureus* (B) on the antimicrobial (GPPS 1.0%; black bar) and non-antimicrobial (GPPS BL; gray bar) surfaces.
The average values are indicated with their standard deviations (n=10-20). The data were taken from the cells incubated for 5 - 10 hours. The experiments repeated two or three times, independently.

Figure 5. Distribution of the colony sizes of *E. coli* and *S. aureus* on the antimicrobial (GPPS 1.0%; black bars) and non-antimicrobial (GPPS BL; gray bars) surfaces at various nutrient concentrations. The data were taken from the cells incubated for 20 - 30 hours. The experiments were repeated two or three times, independently.