A Scanning Electron Microscopic Method for the Study of Bacterial Growth Inhibition by the Paper Disc Method

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Summary
We previously found an inverse correlation between the number of bacteria and the number of fungi collected throughout the year from tatami mats in judo halls. Antibacterial substances produced by the fungi are presumably responsible for the decline in bacterial populations. Culture extracts from fungi isolated from the tatami mats were prepared and their antibacterial activity against Micrococcus luteus, Staphylococcus warneri, and Bacillus subtilis isolated from the mats was determined by the paper disc method. For all 3 bacteria, clear inhibitory zones were obtained indicative of the presence of a growth inhibitory compound in the fungal culture extract. A sampling method was developed for the scanning electron microscopic observation of bacteria around the inhibitory zones produced by the fungal culture extract. Using this method, changes in the surface microstructures of bacteria existing inside the inhibitory zone and growth region were successfully compared by scanning electron microscopy.

Key words Bacteria, Fungi, Inhibitory zones in antibacterial assays, Scanning electron microscope.

The microbial flora, the number of bacteria and the number of fungi on the tatami mats of the judo hall in Nippon Sport Science University change during the course of a year. The number of bacteria on the tatami mats decreases when the number of fungi increases, and the number of bacteria increases when the number of fungi decreases (Nakamura 2006). This inverse correlation between the number of bacteria and fungi is presumably caused not only by mutual competition for nutrients but also by antibacterial compounds produced during fungal growth. The paper disc method, a simple inexpensive method for determining the antibacterial activity of culture extracts, was used to show that bacteria frequently isolated from the tatami mats of the judo hall are sensitive to compounds in extracts prepared from fungi isolated from the tatami mats. A newly developed sampling method for the scanning electron microscopic analysis of bacteria within the fungal extract inhibitory zone has allowed a comparison of the surface microstructure of bacteria inside the inhibitory zone and those within the growth region.

Materials and methods

Freeze-drying of fungal culture
The fungi isolated from the vinyl chloride tatami mats in the judo hall of Nippon Sport Science University were pure-cultured, followed by shake culture in Sabouraud’s liquid medium at 27°C for 12 weeks (Nara et al. 2007). Fungi were separated from the culture fluid by centrifugation at 3,000 rpm for 15 min. The culture fluid was filtered through a 0.22-μm membrane (Millipore,
Billerica, Ma, USA), frozen at \(-80^\circ\text{C}\), and converted to a powder by freeze-drying (Labconco Model 75035, Kansas City, Mo. USA) for 2 d (Harris 1954, Rowe 1970, Ishida and Fukushima 2008). The freeze-dried culture fluid was reconstituted with 0.1 volume of distilled water and this 10-fold concentrated fungal culture fluid was used in the antibacterial paper disc assays.

**Bacterial species tested**

*Micrococcus luteus*, *Staphylococcus warneri*, and *Bacillus subtilis* were isolated at various times throughout the year (Nara and Kiyohara 2008) directly from the vinyl chloride tatami mats in the judo hall. Bacterial sensitivity to antibacterial compounds in the 10-fold concentrated fungal culture fluid was determined using the paper disc method.

**Antibacterial assay using the paper disc method**

Bacteria were cultured on Mueller-Hinton agar medium. For the antibacterial paper disc assays, 80 \(\mu\)l of 10-fold concentrated fungal culture was dropped onto a 10 mm diameter paper disk (Advantec, Tokyo, Japan). The disc was placed onto a Petri dish containing Mueller-Hinton agar medium that had been inoculated with test bacteria. The plates were incubated for 24 h at 37°C and the presence of inhibition zones was determined (Horino 1973, Uechi et al. 2000).

**Sampling method for SEM**

To prepare inhibitory zones for scanning electron microscopy, 15 ml of 2.5% glutaraldehyde was poured directly onto the agar medium and fixation was allowed to proceed at 4°C for 2 h. The fixative solution was removed with a pipette and the plates were washed 4 times for 15 min with 0.1-M phosphate buffer which was poured over the plate and removed with a pipette. After the fourth phosphate wash, the samples were dehydrated by incubation at 4°C for 10 min in an ascending ethanol series (50%, 60%, 70%, 80%) followed by incubation for 10 min in 90% ethanol at room temperature. Agar slices of approximately \(5\times7\) mm were excised with a double-edged razor from the inhibitory zones and they were cut to a thickness of about 2 mm (height). The slices were incubated 3 times for 15 min at room temperature in 100% ethanol followed by incubation 3 times for 15 min in isoamyl acetate. The slices were removed from the isoamyl acetate and dried in liquefied carbon dioxide with a critical-point drier (JCPD-5, Jeol, Tokyo, Japan). After drying, the samples were platinum-coated at 30 mV for 90 s with an AUTO FINE COATER (JFC-1600, Jeol, Tokyo, Japan) and examined in a scanning electron microscope (JSM-6460LV, Jeol, Tokyo, Japan) (Osafune et al. 2006).

**Results and discussion**

Growth of *Micrococcus luteus*, *Staphylococcus warneri*, and *Bacillus subtilis* was inhibited in the antibacterial paper disc assay by a 10-fold concentrated fungal culture fluid. Examination of the *Micrococcus* inhibitory zone by SEM shows a clear demarcation between the bacterial growth zone (Fig. 1(A), left) and the inhibitory zone (Fig. 1(A), right). A dense spherical bacterial population is seen in the growth zone with just a few clusters of bacteria at the edge of the inhibitory zone. Few if any bacteria are seen within the inhibitory zone (Fig. 1(A), right). At high magnification, the *Micrococcus* within the growth zone (Fig. 1(B)) are seen to have a uniform spherical surface while the few bacteria within the inhibitory zone have an irregular granular surface (Fig. 1(C)).

SEM analysis of the inhibitory zone produced when *Staphylococcus warneri* is exposed to 10-fold concentrated fungal culture fluid in the antibacterial paper disc assay shows an area of extensive bacterial growth (Fig. 2(A), left) separated by a sharp boundary from the inhibitory zone which is virtually devoid of bacteria (Fig. 2(A), right). The growing bacteria observed at high magnification appear as smooth surfaced discs (Fig. 2(B)). The few bacteria found in the inhibitory
zone appear shrunken and have irregular structures of variable size on their surface (Fig. 2(C)).

*Bacillus subtilis* is a spore forming rod shaped bacteria whose vegetative growth is inhibited in the filter paper disc assay by a 10-fold concentrated fungal culture extract. SEM analysis shows that the growth zone is composed of a dense mat of rod shaped vegetative cells (Fig. 3(A), left). The mat of bacteria abruptly ends at the edge of the inhibition zone. Few bacteria are seen within the inhibition zone (Fig. 3(A), right). High magnification SEM shows small spherical structures and numerous tubular projections on the surface of the growing vegetative cells (Fig. 3(B)). Compared to the vegetative cells found in the growing zone, the cells in the inhibitory zone are narrower, they have larger and more numerous spherical structures on their surface and they lack the tubular projections found on the cells in the growing zone (Fig. 3(C)).

Fig. 1. (A) SEM image of the inhibitory zone formed in the filter paper disc assay when *Micrococcus luteus* isolated from the vinyl chloride tatami mats in the judo hall were exposed to a fungal culture extract produced by a fungus isolated from the same vinyl chloride tatami mats. The zone of actively growing bacteria is seen on the left separated by a sharp border from the growth inhibition zone on the right. (B) High magnification SEM of the bacteria in the growth region. (C) High magnification SEM of the bacteria in the inhibitory zone.
The filter paper disc assay showed that a fungal culture extract produced by a fungus isolated from the vinyl chloride tatami mats in the judo hall inhibited the growth of 3 bacterial species isolated from the mats. This indicates that the inverse relationship between bacterial and fungal populations on the mats results from antibacterial agents produced by the fungi. A newly developed SEM fixation protocol allowed the surface structure of the bacteria to be examined in the growth inhibitory zone and in the growing zone. For all 3 bacteria studied, there were marked changes in the surface structure of the bacteria in the inhibitory zone. The most marked structural change was the appearance of rough tubular and/or spherical surface patches suggesting that the fungal extract disrupts carbohydrate deposition on the cell wall and/or capsule leading to cell death.

Fig. 2. (A) SEM image of the inhibitory zone formed in the filter paper disc assay when Staphylococcus warneri isolated from the vinyl chloride tatami mats in the judo hall were exposed to a fungal culture extract produced by a fungus isolated from the same vinyl chloride tatami mats. The zone of actively growing bacteria is seen on the left separated by a sharp border from the growth inhibition zone on the right. (B) High magnification SEM of the bacteria in the growth region. (C) High magnification SEM of the bacteria in the inhibitory zone.
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