Three-Dimensional Observation of Soil Bacteria in Organic Debris with a Confocal Laser Scanning Microscope*

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Confocal laser scanning microscopy (CLSM) was applied to three-dimensional (3-D) observation of bacteria harboring in soil organic matters. Ethidium bromide (EB) was employed for staining soil bacteria, since EB did not stain strongly soil constituents other than bacteria. Staining of soil specimens in 100 μg/ml of EB at pH 7.2 for 3 min followed by observation under blue excitation offered a satisfactory preparation for CLSM. A sequence of 60 confocal images of a specimen (soil organic debris 18 μm thick) was obtained by optical sectioning at different levels in the specimen at intervals of 0.3 μm. From the 60 sections, several types of 3-D projections were generated by image processing: a micrograph with a great depth (18 μm) of focus, a pair of split stereo images which could be observed stereoscopically with the naked eye, etc. These 3-D images enabled to observe the spatial distribution of bacteria in organic debris in detail including bacterial colonization inside the organic debris. Possible use of CLSM in soil microbiology was discussed.

Key Words: Direct counting, Ethidium bromide, Laser scanning microscopy, Soil bacteria, 3-D observation

Introduction

Confocal laser scanning microscopy (CLSM) has been developed in the last decade. Confocal imaging6 of specimens stained with fluorescent dyes, together with a laser scanning technique, gives a higher resolution than conventional fluorescence microscopy. Furthermore, a confocal pinhole located before a photo multiplier almost completely cuts the fluorescence from off-focus specimen planes and gives an extremely reduced depth of focus for CLSM. The unique mechanism of CLSM, therefore, enables to carry out direct optical sectioning. As a result, CLSM is a useful tool for studying the three-dimensional (3-D) morphology of biological structures by the aid of image processing. Its application in biology has been reported including 3-D observation of neurons from a lamprey5, chromatin in mouse neuroblastoma cells31, and giant chromosomes of a midge Chironomus13.

In this paper, CLSM was used for the 3-D observation of bacteria colonizing soil organic debris. Ethidium bromide (EB), which was applied by Roser (1980)17 to soil microbiology as a fluorochrome for staining soil bacteria, was used with some modification. Since the dye stains well soil bacteria but hardly soil minerals or soil organic debris, it could become a suitable marker for discriminating soil bacteria.

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Materials and Methods

Soil
A pasture soil was sampled from an experimental field at the National Grassland Research Institute, Nishinasuno, northern Japan. The soil belongs to the Nasu volcanic ash soil (light clay) with pH (H₂O) 4.4. Total C and N contents were 5.3% and 0.35%, respectively. A paddy field soil was also collected from an experimental field at the Kyushu National Agricultural Experiment Station, Chikugo, southern Japan. The soil belongs to the Gray lowland soil (light clay with pH 5.7). Total C and N contents were 1.7% and 0.18%, respectively.

Staining procedures
The Nuclepore filter technique of Hobbie (1977) was used for staining the soil suspension with EB (Sigma) to examine the staining conditions (excitation filters, concentration and pH of EB solutions). Soil (1 g) was added to 100 ml of distilled water and homogenized in a blender with stainless steel blades (Ace homogenizer, Nihonseiki Ltd.) at 15,000 rpm for 5 min. The soil suspension was further diluted 10-fold (1,000-fold as a final concentration) with distilled water. One ml of EB solution (200 µg/ml in 0.1 M phosphate buffer, pH 7.2, unless otherwise noted) was added to a test tube containing 1 ml of 1,000-fold soil suspension (100 µg/ml of EB as a final concentration). The mixture was incubated for 3 min at room temperature, then filtered through a Nuclepore filter (black filter with 0.2 µm pore size, 25 mm diameter) on a vacuum filter holder. Then the filter was rinsed with several drops of 0.1 M phosphate buffer (pH 7.2) and mounted on a microscope slide. One drop of immersion oil (non-fluorescence, nd=1.516, Olympus) was put on the filter and a cover slip was placed on top.

To examine the effects of pH of EB solutions, Teorell and Stenhagen’s citrate-phosphate-borate buffer was used to prepare EB solutions at various pH values ranging from 5.0 to 9.0.

For the 3-D observation by CLSM, several fragments of soil organic debris (≤1 mm size) were picked up with tweezers from soil, then transferred onto a cellulose membrane filter (1 µm pore, 25 mm diameter) on a vacuum filter holder and stained with several drops of 100 µg/ml of EB (pH 7.2). The dye solution was removed under vacuum and the specimens were washed with several drops of 0.1 M phosphate buffer (pH 7.2) under vacuum. The stained materials were transferred onto a microscope slide where one drop of immersion oil was added and the organic matters were scattered in the oil using tweezers. A cover slip was placed on top.

As laboratory distilled water usually contains many bacteria, water for the dilution of the samples as well as the EB solutions was filtered through 0.2 µm filters before use to avoid bacterial contamination. Also, all the apparatus used for staining was washed with filtered distilled water before use.

EB should be handled with care as it is known to be a carcinogen and mutagen.

Conventional fluorescence microscopy
Filter preparations stained with EB were examined with a Zeiss fluorescence microscope (ICM 405) fitted with a Neofluar objective (100×, NA=1.30). Excitation of EB in stained specimens was provided by radiation from a 100 W mercury lamp passed through Zeiss filter cubes: No.01 (UV excitation, 353-377 nm excitation, transmits >397 nm); No.05 (blue-violet excitation, 395-440 nm, >470 nm); No.09 (blue excitation, 450-490 nm, >520 nm); No.15 (green excitation, 540-552 nm, >590 nm). Soil bacteria were counted for at least 10 randomly selected microscope fields in each filter preparation using an eyepiece micrometer disk (5×5 grids). Three replicat-ed filters were prepared for each treatment
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(such as different pH values, concentrations of EB). The means and standard deviations were calculated.

CLSM

A Zeiss laser scanning microscope (LSM 410, Fig.1) equipped with an Argon ion laser (488 nm/10 mW), an LP 570 emission filter, and a high resolution objective (Plan-Apochromat, 63×, NA=1.40) was used for the 3-D observation of soil bacteria in organic debris. Under these conditions, the resolution of the CLSM was estimated to be approximately 0.2 μm in a plane perpendicular to the optical axis, and depth discrimination was calculated to be approximately 0.3 μm. The CLSM, in addition, has a transmit detector for differential interference observation of a specimen.

A sequence of sixty confocal images was obtained by optical sectioning at different levels in the specimen at intervals of 0.3 μm (depth of 18 μm in total) under microprocessor control. Stereoscopic views were generated from the sequence of CLSM pictures by image processing.

Results

Experiments on staining and observation with EB

Procedures for staining and observation with EB were examined in detail using a conventional fluorescence microscope since there are few studies on the use of EB as a stain for soil bacteria.

When the soil suspensions were stained with EB (pH 7.2), stained soil bacteria could be observed under a wide range of excitations.
Table 1  Counts of soil bacteria stained with EB and observed under various excitation wave lengths.

<table>
<thead>
<tr>
<th>Excitation</th>
<th>Chikugo paddy soil</th>
<th>Nishinasuno pasture soil</th>
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<tbody>
<tr>
<td>Blue</td>
<td>1.57±0.11 (100) b)</td>
<td>1.29±0.09 (100)</td>
</tr>
<tr>
<td>Blue-Violet</td>
<td>1.07±0.10 (68)</td>
<td>1.33±0.09 (103)</td>
</tr>
<tr>
<td>Green</td>
<td>1.06±0.10 (68)</td>
<td>1.24±0.07 (96)</td>
</tr>
<tr>
<td>UV</td>
<td>1.03±0.12 (66)</td>
<td>1.03±0.16 (80)</td>
</tr>
</tbody>
</table>

1) For excitation and transmission wave lengths, see Materials and Methods.
2) Obtained by staining soil suspension in 100 μ g/ml EB, pH 7.2, and the count was expressed as a mean ± standard deviation for 3 replicated filters from each soil sample.
3) Values in parentheses show ratios to the counts under blue excitation (=100).

Table 2  Effect of pH of the EB solution on the counts of soil bacteria.

<table>
<thead>
<tr>
<th>pH</th>
<th>Total bacterial counts (×10^6 cells/g dry soil)</th>
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<tbody>
<tr>
<td></td>
<td>Chikugo paddy soil</td>
</tr>
<tr>
<td>5.0</td>
<td>1.31±0.08 (84) b)</td>
</tr>
<tr>
<td>6.0</td>
<td>1.54±0.10 b) (98)</td>
</tr>
<tr>
<td>7.2</td>
<td>1.57±0.11 b) (100)</td>
</tr>
<tr>
<td>8.0</td>
<td>1.52±0.08 b) (97)</td>
</tr>
<tr>
<td>9.0</td>
<td>1.49±0.09 b) (95)</td>
</tr>
</tbody>
</table>

1) Expressed as a mean ± standard deviation for 3 replicated filters from each soil sample.
2) Means within a column followed by the same letter are not significantly different (t-test, p<0.05).
3) Values in parentheses show ratios to the count at pH 7.2 (=100).

Soil bacteria gave an orange fluorescence under blue, blue-violet, or UV excitations, whereas green excitation resulted in red fluorescence. Adsorption of EB onto clay minerals was almost negligible under every excitation examined. Soil organic debris sometimes adsorbed EB and gave a red-orange fluorescence under blue, blue-violet, or UV excitations. Under the blue excitation, however, soil bacteria could be easily distinguished because of their strong orange fluorescence. In contrast, under the blue-violet excitation, bacterial fluorescence was so weak that the discrimination of soil bacteria from soil organic debris was occasionally difficult. Under the UV excitation, some organic debris showed a noticeable yellow-orange autofluorescence which interfered with the observation of bacterial cells. Green excitation yielded the most intense fluorescence of bacteria among the four excitations examined. However, many soil organic debris also gave a red fluorescence, making bacterial observation difficult especially when bacteria colonized such organic debris.

Bacterial counts using EB were compared among various wave lengths of excitation (Table 1). The blue excitation gave bacterial counts equal to or higher than those obtained under the other excitations. The UV excitation yielded the lowest counts in both soil types apparently due to the autofluorescence of organic matters.

Soil suspensions were stained with various concentrations (5-500 μg/ml) of EB at pH.
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Fig. 2 Conventional fluorescence microscopy of bacteria in Chikugo paddy soil stained with EB and filtered on a Nuclepore filter (Blue excitation).

Fig. 4 Laser scanning microscopy of organic debris in Nishinasuno pasture soil stained with EB: A, differential interference image; B, picture with an extended depth (18 μm) of focus which was obtained by overlaying a series of sixty optical sections, showing bacterial colonization of the organic debris (pseudo color used); C, colored image showing the depth information; D, red/green stereo image which can be observed stereoscopically with red/green spectacles.
7.2 and observed under blue excitation. Good fluorescent staining of bacterial cells with minimum staining of soil organic debris was achieved by staining soil suspensions in 50-100 µg/ml of EB for 3 min or more. Low concentrations (5-20 µg/ml) provided a low contrast between bacteria and soil particles, while high concentrations (200-500 µg/ml) led to over-staining of soil organic debris.

In cases where the pH of the EB solutions varied (Table 2), almost the same counts of bacteria were obtained between pH 6 and 9, whereas significantly (p < 0.05) low counts were obtained at pH 5 in both paddy and pasture soils. The fluorescence of stained specimens did not decrease appreciably even after 4 min between pH 7.2 and 8, whereas the quenching of fluorescence was faster and the observation could not be continued for a period of more than 3 min at a pH lower than 7.2 or higher than 8.

Based on the above results, the following procedure was recommended: staining with 100 µg/ml EB at pH 7.2 for 3 min and observation under a fluorescence microscope with blue excitation (450-490 nm, > 520 nm).

Fig. 2 shows a micrograph of a sample from Chikugo paddy soil stained with EB according to the above procedure (Neofluar objective 100 X, Kodak Ektachrome 400 film, 6 s exposure). Soil bacteria gave a bright orange fluorescence with a remarkable contrast with soil particles.

3-D observation by CLSM

To test confocal imaging, an EB-stained soil specimen on a Nuclepore filter was observed with a Zeiss laser scanning microscope LSM 410 equipped with an Argon 488 nm laser. Fig. 3 shows a pair of micrographs of a non-confocal image (Fig. 3A) and a confocal image (Fig. 3B) of the same microscopic field. The micrographs revealed that the confocal image provided a higher resolution by eliminating fluorescence from off-focus specimen planes. Conventional fluorescence microscopy, naturally, gives non-confocal pictures (Fig. 2).

Fig. 3 Laser scanning microscopy of bacteria in Chikugo paddy soil stained with EB and scanned with Ar laser 488 nm: A, non-confocal; B, confocal.
sections thus obtained were digitized and stored in a host computer as well as in a magneto optical disk.

The 60 images of a section series were processed in various manners to obtain 3-D representations of the specimen. Firstly, the 60 pictures were overlaid to produce an image with a great depth of focus (Fig. 4 B). A picture showing bacterial colonization of a piece of organic debris with a remarkable depth of focus (18 μm) was obtained. Secondly, the projection was colored to visualize the depth information (Fig. 4 C: red represents front planes and blue rear planes). Thirdly, a stereoscopic image was generated from the 60 sections as a red/green stereo image which could be viewed stereoscopically with red/green spectacles (Fig. 4D). Fourthly, an alternative representation of 3-D images was created as a pair of split stereomicrographs (Fig. 5) which could be observed stereoscopically with the naked eye. In the stereomicrographs, as shown in Fig. 4 D and 5, bacterial distribution could be observed in detail; some bacteria attached to the surface of the organic debris (Fig. 5, a) while others were present inside the organic debris (Fig. 5, b). The surface of the organic debris appeared as a weakly stained structure.

Finally, the projection was rotated along the axis Y with an angle of 0°, 30°, and 60° (Fig. 6 A-C). In these pictures, the contrast was enhanced to facilitate the observation of bacteria. The lateral view (Fig. 6 C) revealed that fewer bacteria colonized the back of the organic debris (right side of the micrograph) than the front (left side).

Discussion

For staining soil bacteria, many fluorochromes have been used including fluorescein isothiocyanate, europium chelate, acridine orange, and ethidium bromide (EB). In preliminary experiments, it was observed that EB was superior to the other fluorochromes since EB stained specifically soil bacteria due to its high affinity to nucleic substances. Thus the discrimination of bacteria from nonbacterial soil particles was easy compared with the use of other fluorochromes (Details will be published elsewhere).

EB was first applied to soil microbiology by Roser (1980) as a stain for bacteria and fungi and then used for various natural samples including pond sediment by Swannell and Williamson (1988). Staining and observation procedures with EB, however, had not been examined in detail for the purpose of direct observation of soil bacteria.

In the above studies, EB was used in distilled
water (thus not bufferized) and observed under blue-violet excitation (390–490 nm excitation, transmits > 510 nm) or used at pH 9.2 and observed under green excitation (530–560 nm, > 580). The present study, however, showed that staining at pH 7.2–8.0 followed by observation under blue excitation (450–490 nm, > 520 nm) gave better results for the soil samples used.

Laser scanning microscopy, in combination with appropriate fluorescence technique, enables to obtain a series of optical sections with high resolution from thick soil organic constituents as demonstrated in the present paper. These results indicate that CLSM is a suitable tool for observing spatial interrelationships between soil microorganisms and other soil constituents such as organic matters, plant roots, or clay particles.

Rotated images of organic debris (Fig. 6) showed that fewer bacteria colonized the back of the organic debris than the front. This was probably due to the fact that the organic debris were not enough transparent to pass through the fluorescence from bacteria located in deeper areas. It appears that specimens thinner than about 10 μm may give clear 3-D images under the present conditions.

Scanning electron microscopy (SEM) also allows 3-D observation and has been applied to soil microbiology. Researchers, however, have often encountered difficulties with SEM; clay minerals are occasionally indistinguishable from soil bacteria, since many clay particles resemble bacteria in size and shape. These difficulties can be overcome by using CLSM in combination with EB-staining as indicated in the present paper.

Another possible use of CLSM in soil microbiology would be the automatic measurement of microbial biomass in soil. Conventional fluorescence microscopy offers micrographs showing a fluorescence from off-focus specimen planes which disturbs the accurate estimation of cell size by image analysis. In contrast, CLSM gives micrographs of soil bacteria with a great depth

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Fig. 6 Images obtained after rotation along the axis Y: A, angle of rotation 0° (the same image as shown in Fig. 4B but contrast was enhanced to facilitate the observation of bacteria); B, 30°; C, 60°.
of focus which may enable to perform automatic image analysis with greater accuracy for obtaining bacterial biovolume and thus soil bacterial biomass.

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References