Rapid In Situ Enumeration of Physiologically Active Bacteria in River Waters using Fluorescent Probes

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In situ enumeration methods utilizing fluorescent probes were used to estimate the number of physiologically active bacteria in river water. Two fluorogenic compounds, 5-cyano-2,3-ditolyl tetrazolium chloride (CTC) and 6-carboxy fluorescein diacetate (6CFDA), were chosen for direct epifluorescent microscopic detection of active bacteria. CTC is a soluble redox indicator which is reduced by respiring bacteria to fluorescent CTC-formazan crystals. 6CFDA is hydrolyzed by nonspecific esterases to produce the fluorescent compound, 6-carboxy fluorescein. Estimates of the number of active bacteria identified by these fluorescent probes were compared with those obtained with the plate count method. Bacteria with respiratory activity, as determined by CTC reduction, accounted for approximately 10% of the total bacteria at oligotrophic sites and 15 to 20% at eutrophic sites. These values exceeded those obtained with the plate count method. Estimates of enzymatically active bacteria, as determined by 6CFDA hydrolysis, were also higher than those obtained with the plate count method, constituting 40 to 50% of the total bacteria at both oligotrophic and eutrophic sites. These results indicate that some non-culturable bacteria retain physiologic activity and may play an important role in the ecosystem.

Key words: fluorescent staining, respiratory activity, esterase-activity, river

Introduction

Naturally occurring bacteria in the environment have various functions as biogeochemical components of the ecosystem. Those bacteria which are particularly physiologically active are the most important. Although plate count methods have often been employed to enumerate and characterize physiologically active bacteria\(^1,11,15,28\), these methods underestimate the actual active population because certain bacteria from the environment do not form colonies on established media. Therefore, we developed a system for the accurate identification of physiologically active bacteria in their natural environment.

Microautoradiography\(^{13,22}\) allows quantification of the metabolic activity of bacterial cells, however its application is limited by the high background noise and the requirement for a radioisotope. An in situ technique, the direct viable counting method (DVC), allows the enumeration of bacteria which have the ability to divide\(^9,10\); however, it can not be used with bacteria resistant to nalidixic acid. An alternative method using fluorogenic esters such as fluorescein diacetate (FDA) has been reported\(^1,6,8\). FDA is normally a non-fluorescent, non-polar compound that readily penetrates cell membranes. Intracellular FDA is hydrolyzed by non-specific esterases, if present and active, resulting in the release of fluores-
cein. Fluorescein, which fluoresces brilliant green when irradiated with blue light, is polar and is retained in the cell. Although the use of FDA has been reported by several workers, it has a limited application because once cleaved, the released fluorescein is poorly retained by bacterial cells\textsuperscript{3,7}. The 6-carboxymodification of FDA to 6 CFDA (6-carboxy fluorescein diacetate) allows better cell retention\textsuperscript{5,23} and thus stains a wider variety of bacteria. 6 CFDA was chosen for the detection of esterase active bacteria in this study. Similarly, a tetrazolium salt, 5-cyano-2,3-ditolyl tetrazolium chloride (CTC), which is reduced by bacterial respiration to water-insoluble CTC-formazan, has been employed as an indicator of bacterial respiratory function\textsuperscript{17,19,20}. CTC-formazan fluoresces bright red under blue light-excitation. Counterstaining of 6 CFDA-, or CTC-stained samples with the DNA-specific fluorochrome 4',6-diamidino-2-phenylindole (DAPI), which is excited under UV light, allows enumeration of both the total bacterial population as well as the active sub-population within the same preparation.

We determined the proportion of bacteria with enzymatic and respiratory activity in the natural aquatic environment at the single cell level using these double-staining techniques, and compared our results with those obtained with the plate count method. We then examined the relationship between bacterial physiologic function and the environment.

**Materials and Methods**

**Sampling stations**

River water was collected at four sites (Takayama, Takiue, Kuwazu and Kitahashi) in the northern part of Osaka, Japan (Fig. 1). The sampling period was from September 27, 1994 to February 2, 1995. The Minoh river originates in the Minoh Mountains and joins the Ina River about 1.5 km from the Kuwazu bridge. Takiue is located in the Minoh National Park. At this point, the river is narrow, shallow and fast flowing. The stream bed is very rocky and the water is exposed to neither domestic nor industrial effluents. Takayama is located upstream of Takiue and the environment is similar. Both Takayama and Takiue are considered unpolluted sites. Kuwazu of the Ina River is located in an industrial area and considered polluted\textsuperscript{14,16}. At this point, the river is comparatively wider. Kitahashi is located in a commercial area, Osaka Business Park, and considered highly polluted. Domestic water flows into this river upstream. Water samples were collected from the surface at each of the four sites. Ambient temperature (A.T.), water temperature (W.T), pH and electrical conductivity (EC) were measured on site. The total organic carbon (TOC) of river water was determined with a TOC analyzer (TOC-500; Shimadzu Co.).

**CFU**

The number of colony forming units (CFU) was determined by spreading dilute river water samples on 0.05 media (0.5 g polypeptone; 0.25 g yeast extract; 0.1 g glucose; 15 g agar per liter H\textsubscript{2}O), which were incubated at 25°C for one week before counting\textsuperscript{20}.

**6 CFDA-DAPI double staining**

Natural river water samples were mixed with a half volume of 6 CFDA buffer (0.3 M phosphate buffer [pH 8.5], \(15\%\) NaCl, 1.5 mM EDTA). 6 CFDA stock solution (Calbiochem Co.; 10 mg/ml in
acetone) and DAPI stock solution (Sigma Co.; 10 μg/ml in sterilized water) were applied (final concentration; 6 CFDA: 150 μg/ml, DAPI: 1 μg/ml) and incubated for 3 min at room temperature under dark conditions. 6 CFDA-DAPI stained bacteria were trapped by vacuum onto a Nuclepore black filter (Costar Scientific Co.; pore size: 0.20 μm). Filters were then air dried and mounted on glass microscope slides with non-fluorescence immersion oil (Olympus Co.).

**CTC-DAPI double staining**

CTC stock solution (Polysciences Inc.; 50 mM in deionized water) and 0.05 media without agar were added to natural river water (final concentration: 1.0 mM and 10% (v/v), respectively). Samples were incubated for 30 min at 25°C under dark conditions with shaking. After incubation, the samples were counterstained with DAPI (final concentration; 1 μg/ml) for 3 min. Stained bacteria were trapped onto Nuclepore black filters and examined by epifluorescence microscopy as well as 6 CFDA-DAPI double-staining.

**Epifluorescence microscopy and enumeration**

A BHS-RFK epifluorescence microscope (Olympus Co.) equipped with a 100-W mercury burner was used to enumerate bacterial cells. The filter combination for viewing CTC-, or 6 CFDA-treated preparations consisted of an excitation filter (BP490+ EY455), a dichroic mirror (DM500) and an absorption filter (O515). DAPI stained bacteria could be viewed with an excitation filter (UG1), a dichroic mirror (DM400) and an absorption filter (L420); thus, physiologically active bacteria and all bacteria were enumerated under blue and UV excitation respectively. Bacterial number was adjusted to more than 80 cells per field. Approximately 30 fields per sample were counted.

**Results and Discussion**

Optimal conditions for observation and enumeration of bacteria with respiratory or enzymatic activity were investigated with bacteria sampled at Kitahashi. 6 CFDA is a fluorogenic ester which has the ability to detect esterase activity in cells. However, high background in microscopic fields with 6 CFDA remained a problem. We found this problem could be overcome by drying the filter after trapping bacteria and limiting oil immersion during mounting. 6 CFDA-stained bacteria then appear bright green and become easy to count. Different incubation times for staining esterase active bacteria with fluorogenic esters have been reported\(^2,4,26\). Thus, the effect of incubation time on bacterial fluorescence was investigated by measuring the percentage of esterase active cells to total cells. Incubation for 3 min to 60 min did not result in improved staining; thus, 3 min was selected as the optimum. The optimum concentration of sodium ion in 6 CFDA buffer was also investigated, and the best results were obtained by adding 5% NaCl for staining. Suitable conditions for CTC-DAPI double-staining were examined as well. Rodriguez et al. suggested that the lack of carbon substrates in water samples limited the in situ respiratory activity of bacteria\(^17\). Supplementation of river water samples with 0.05 media was performed for effective CTC-reduction but prevented total direct counts (TDC) from increasing during incubation. The most favorable concentration and time for reduction was 1.0 mM CTC and a 30 min incubation.

After staining with 6 CFDA and DAPI for 3 min, river water bacteria with esterase activity fluoresced green by epifluorescence microscopy when irradiated with blue light (Fig. 2a). Ultra-violet irradiation caused all bacteria to fluoresce brilliant blue (Fig. 2b). Respiring cells reduced CTC to crystalline CTC-formazan, which could be visualized as red crystals inside the bacteria with blue light irradiation (Fig. 3a), and all bacteria were also visible with UV optics and epi-illumination (Fig. 3b).

Table 1 shows physicochemical water quality of the four river water specimens collected between Sep. 1994 and Feb. 1995. These values confirm that Takayama and Takiue are unpolluted, and Kuwazu and Kitahashi are polluted. Fig. 4 shows the number of total and physiologically active bacteria at four sites during this sampling period. TDC values were
Fig. 2. Epifluorescence micrographs of bacterial cells double-stained with 6CFDA and DAPI

- a) B-excitation
- b) UV-excitation

Fig. 3. Epifluorescence micrographs of bacterial cells double-stained with CTC and DAPI

- a) B-excitation
- b) UV-excitation
always higher at Kuwazu and Kitahashi. The number of bacteria with respiratory activity by fluorescent probe was almost always higher than that of viable bacteria with the plate count method and less than the number of bacteria with enzymatic activity.

Fig. 5 shows the percent of active bacteria to total

<table>
<thead>
<tr>
<th>Sampling stations</th>
<th>A.T. (°C)</th>
<th>W.T. (°C)</th>
<th>pH</th>
<th>EC (μS/cm)</th>
<th>TOC (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Takayama (n=7)</td>
<td>10.0±6.0*</td>
<td>9.6±5.1</td>
<td>8.1±0.3</td>
<td>136±16</td>
<td>1.0±0.6</td>
</tr>
<tr>
<td>Takiue (n=13)</td>
<td>13.5±5.7</td>
<td>13.7±5.6</td>
<td>8.0±0.2</td>
<td>157±14</td>
<td>1.8±1.0</td>
</tr>
<tr>
<td>Kuwazu (n=8)</td>
<td>16.9±7.0</td>
<td>18.5±4.9</td>
<td>7.6±0.2</td>
<td>503±89</td>
<td>6.4±3.9</td>
</tr>
<tr>
<td>Kitahashi (n=15)</td>
<td>14.6±7.3</td>
<td>16.8±5.3</td>
<td>7.5±0.2</td>
<td>541±157</td>
<td>6.7±2.6</td>
</tr>
</tbody>
</table>

* Values: Means±SD.
bacteria in these samples. CFU averaged 3% of total bacteria at Takayama, 1% at Takiue, 6% at Kuwazu and 18% at Kitahashi. CTC-stained bacteria comprised 8% of total bacteria on average at Takayama, 9% at Takiue, 15% at Kuwazu and 20% at Kitahashi. These results showed that higher percentages were obtained in polluted sites. 6 CFDA-stained bacteria was 39% of total bacteria on average at Takayama, 50% at Takiue, 44% at Kuwazu and 46% at Kitahashi. These values showed little variation in relation to the pollution level of the sampling sites.

Generally, it is very difficult to make all bacteria in a natural sample form colonies, because their auxotrophy is variable12,18). In addition, the quantitative measurement of aggregate populations presents problems when using the plate count methods. Techniques for staining viable bacteria with specific fluorochromes and epifluorescence microscopy overcome these disadvantages. In the present study, CTC and 6 CFDA were used to enumerate physiologically active bacteria in river water at various locations with different levels of pollution. Plate counting was also reconfirmed to underestimate the number of respiratory and enzymatically active bacteria in the natural aquatic environment. The difference between the number of CTC reduced cells and colony formed cells is similar to that in reports concerning bacteria in drinking water, indigenous soil, and biofilm tested by tetrazolium reduction20,25,29), however, the difference was found to be smaller at more eutrophic sites. This indicates that CTC staining is particularly useful for the detection of respiring bacteria in those environments in which plate count methods miss a high percentage of the active bacteria actually present, for example, oligotrophic sites. Tabor and Neihof have measured respiratory active bacteria with an INT reduction system and reported that the percentage of respiring bacteria to total bacteria ranged from 5 to 36% in freshwater systems and 4 to 61% in the marine environment21). On the other hand, Winding et al. reported that respiring bacteria accounted for 2 to 6% of total bacteria in soil25). The fraction of respiring bacteria seems to vary with the environment.

"Non-culturable bacteria", that is, those without growth potential in media commonly used, retain physiological activity in the natural aquatic environment regardless of the pollution level (Fig. 4, 5), and may play important roles in the ecosystem. The difference in the level of respiratory and enzymatic activity of bacteria in river waters suggests that even resident bacteria without respiratory activity retain enzymatic activity. Estimates by 6 CFDA-staining de-
pend on enzymatic activity and may show high values even if bacteria lose their growth potential and respiratory activity. Thus, as the number of 6 CFDA-stained bacteria may sometimes include bacteria without growth potential, it is better to use several methods for evaluating bacterial physiological activity.

One useful technique to analyze the fraction of physiologically active bacteria without growth potential may be the use of fluorescent in situ hybridization which allows the detection of specific bacteria with a target sequence. Analysis of the composition of bacterial populations by fluorescent in situ hybridization, combined with specific fluorochromes as vital stains, might reveal populations with physiological activity in the ecosystem at the genus or species level.

CTC-DAPI and 6 CFDA-DAPI double-staining expedite data collection and provide results faster than the conventional methods; thus these double-staining techniques would be also suitable for analysis of food and water hygiene as well as hospital sanitation.

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References

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