Rapid and Multiple In Situ Identification and Analyses of Physiological Status of Specific Bacteria Based on Fluorescence In Situ Hybridization

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Running title: In Situ Analysis of Physiology of Specific Bacteria

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Abstract

Quantitative analysis of the target microorganism in microbial communities is important for the assessment of bacterial activity in environment. Here we present a method of a combination of fluorescence in situ hybridization (FISH) method and live/dead staining which allows in situ identification and analysis of physiological status of specific bacteria.

Keywords

Fluorescent in situ hybridization, live/dead staining, fluorospheres, physiological status

Monitoring the physiological status of microbial communities provides useful data for studies in both applied and basic environmental science (1-3). The live/dead fluorescent dyes such as Oregon Green and propidium iodide are widely used to analyze the physiological status of microbial cells in communities (4-6). Although live/dead staining allows rapid evaluation of cell viability by different fluorescent signals without strict treatments, this method is not able to identify target cells in a community of bacterial cells of different genera.

To identify target cells in environmental samples, fluorescent in situ hybridization (FISH) with rRNA-specific oligonucleotide probes has been increasingly used (7,8). Although FISH has many advantages, analysis of FISH images still remains a challenge, because the intensity of positive signal is variable even in the same sample. To increase hybridization efficiency, in addition to the original procedure involving formaldehyde or paraformaldehyde fixation (9), organic solvent treatment (10), enzyme treatment (11), and acid hydrolysis treatment (12) have been used. But as far as the technique is dependent on RNA-probe hybridization, the cells must be fixed by any means, which disables live/dead analysis. Since the presence of ribosomal RNA indicates the cellular protein synthesis activity, FISH-labeled cells are regarded as live cells (13,14). But there are also exceptions, wherein ribosomal RNA is present but cells have no biological activity (15). This poses a problem in monitoring the physiological status of target bacterial cells. Savichtcheva et al. reported a method for live/dead staining combined with FISH, but samples for staining and FISH were prepared separately (16). The method does not allow physiological status analysis of

51 the cells which are to be detected by FISH.

52 In this study, we present a method to analyze physiological status of target bacteria.
53 The method comprises evaluation of cellular physiological status with live/dead
54 staining, followed by the FISH procedure to identify the target cells on the same
55 membrane filter. Glue-assisted cell immobilization on membrane filters and
56 modification of the glue-related FISH insufficiency were developed.

57 *E. coli* DH5α was grown at 28°C in liquid Luria-Bertani medium (LB, 10 g/l
58 tryptone, 5 g/l yeast extract, 10 g/l NaCl). *P. putida* KT2440 (pCAR1::rfp) carrying a
59 plasmid pCAR1 containing a reporter gene for red fluorescent protein (pCAR1::rfp)
60 was cultivated in 5-ml 1/3-diluted LB liquid medium (1/3LB, 3.3 g/l tryptone, 1.7 g/l
61 yeast extract, 5.0 g/l NaCl) containing 25 μg/ml kanamycin (17). These cells were
62 harvested, washed twice with phosphate-buffered saline (PBS, 1.64 g/l
63 $Na_2HPO_4 \cdot 7H_2O$, 0.28 g/l $NaH_2PO_4 \cdot 2H_2O$, 8.0 g/l mM NaCl pH 7.4) and resuspended
64 in PBS at 1.0 x 10^7 cells per milliliter. Ten microliter of the cell suspension was
65 collected in 1.5-ml tube containing 1-ml PBS. Then 5 μM Oregon Green 488
66 carboxylic acid diacetate succinimidy ester (Molecular Probes, Eugene, OR) was
67 added and the samples were incubated at 30°C in dark. After 30 min, 3 μM propidium
68 iodide (PI) was added into *E. coli* DH5α sample, and incubated for 3 minutes. *P.*
69 *putida* KT2440 (pCAR1::rfp) was stained with Oregon Green only. Proper amount
70 (about 100 fluorospheres) of Flow-check fluorospheres (Fullerton, Beckman Coulter,
71 Inc. CA) was then added to the samples. The cells and fluorospheres were trapped on
72 a black-colored polycarbonate membrane filter (0.2-μm pore size, 25 mm in diameter,

Toyo Roshi Kaisha, Tokyo, Japan) in vacuo. The filters were wet with 15 μl of 3% glycerol and cut into 3 x 3 mm sections with a razor blade. One membrane filter can be cut into 9 pieces. Eight-well printed glass slides (Matsunami Glass, Osaka, Japan) were cleansed with 75% ethanol and dried prior to use. Samples were prepared for different immobilization procedures which included the glue-assisted method, agar-trap method, and control (without glue or agar). In glue-assisted cell immobilization, each well was coated with 3 μl of low fluorescent waterproof glue (a silylated polyurethane resin-based glue dissolved in isopropyl alcohol (0.01 g/ml), Bond Ultra SU, Konishi, Japan). The glue adhere any materials including bacterial cellular membrane components. After solidification in aerobic condition, the glue firmly adhered even when treated with water or ethanol, and the glue showed little autofluorescence under fluorescent microscopic observation**.** The cut filters were then carefully placed on the wells. The samples were allowed to dry for 30 min in dark (Fig. 1) and appropriate volume of 10% glycerol was added on each well. The samples were observed with fluorescence microscopy after putting cover glasses.

For cell immobilization, agar trap method was also used. The bacterial cells were mixed with 2 ml of 0.1% low melting agar (SeaPlaque Agarose, Cambrex Bio Science Rockland, Inc., USA) and trapped on a membrane filter as described above. After viability check, the filter was air-dried at room temperature and kept at 4°C until use.

Viability of the cells was evaluated with an epifluorescence microscope (BX50, Olympus, Co., Tokyo, Japan) equipped with Olympus DP70 digital camera. All samples were observed with the same exposure time and photographic sensitivity. A

470-490-nm band-pass filter and a 520-550-nm band-pass filter were used to excite Oregon Green and PI, respectively. Live and dead bacteria showed green and red fluorescent signals, respectively. The green fluorescence images were superimposed on the red fluorescence images by using DP Manager Software (Olympus). Eighteen images, each containing approximately 100 cells, were collected to calculate the percentage of live/dead cells.

We compared the cell numbers on the filters before and after FISH treatment with different cell immobilization procedures. In the control experiment using *E. coli* DH5α, only 49±5.5% cells remained on the filter (without the glue immobilization treatment), while 61±4.5% remained by agar trap method, and 94±8.0% remained with the glue (mean±SD, N=10 images). These results suggested that cells could be immobilized efficiently by glue treatment in the FISH procedure. Agar-trap increased immobilization efficiency but adhesive strength is lower than that of glue treatment. We also tested various kinds of glues, but all of which showed high background fluorescence or poor adhesiveness, thus they were inefficient for analysis (data not shown).

By glue treatment, all cells were immobilized tightly on the membrane filter. To enhance uptake of probes by increasing cell membrane permeability, organic solvent treatment (propanol, acetone) (10), enzyme treatment (lysozyme, proteinase K, alkaline protease) (11), and acid hydrolysis treatment (12) have been tested. As a result, fluorescent intensity was increased for 15% by alkaline protease treatment without increasing of any background fluorescence (data not shown). The initial

117 live/dead fluorescent signal was removed by passage through 0.1% sodium
118 hypochlorite for 1 min and rinsing with PBS. After confirming disappearance of the
119 signal, the slides were fixed with 85% ethanol/formalin (9:1 v/v) for 4 h. The slides
120 were rinsed thoroughly with 0.1% Nonidet P-40 to remove formalin and air-dried.
121 Fifty microliters of protease solution (100-fold diluted alkaline protease solution
122 provided in Wizard *plus* SV Minipreps DNA Purification System kit, (Promega
123 Corporation, Madison, WI, USA)) was applied to increase hybridization efficiency
124 and to reduce non-specific binding. Then the samples were dehydrated by successive
125 passages through 50%, 80%, and 95% ethanol (3 min each). Finally 20 μl of 95%
126 ethanol containing 0.1% Nonidet P-40 was dropped on each well of the glass slide and
127 the slides were air-dried at 37 °C.
128 Hybridization was performed in a hybridization buffer (0.9 M NaCl, 20 mM
129 Tris–HCl (pH 7.2), 0.001% sodium dodecyl sulfate (SDS), 10% deionized formamide
130 and probe (10 ng/L) at 47°C for 2.5 h. Then the slides were washed in a buffer (180
131 mM NaCl, 20 mM Tris–HCl (pH 7.2), 5 mM EDTA, and 0.001% SDS) at 47°C for 10
132 min. The samples were subjected to microscopic observation. The presence of
133 fluorospheres enabled proper repeated positioning of microscopic fields and
134 identification of cells to be analyzed.
135 Fig. 2 shows *E. coli* DH5α cells traced by FISH after viability check. As a result, 98
136 ±2.6% were live cells and 95±6.9% cells were labeled with Alexa488- or Cy3-
137 labeled universal bacterial probe EUB 338 (5’-GCTGCCTCCCGTAGGAGT) (18).
138 In alkaline protease treatment, incubation time for *E. coli* DH5α and *P. putida*

KT2440 should be optimized independently, suggesting that this treatment is depended on bacterial species (data not shown). This result suggested that with alkaline protease treatment and the glue-assisted immobilization, cells could be efficiently labeled with FISH probes. Some PI-stained cells assumed to be dead were also labeled with FISH probe, suggesting that FISH results do not accurately represent the physiological status.

In many reports, fluorescent proteins were used to trace target cells in environmental samples, and cells emitting fluorescent signals were considered as live cells (19). In this study, *P. putida* strain KT2440 cells were quantitatively analyzed with our protocol (Fig. 3). Nearly 90% of the initial bacterial cells were recovered after FISH procedure using *Pseudomonas*–specific, Cy3-labeled probe PSU (5'-GCCGCTCTCAAGAGAAGCA). Almost all Oregon Green-stained cells (live cells) were detected by FISH (yellow arrows). Two to three percent of live cells showing no RFP signal were also detected by FISH. Ninety percent of dead cells, which were not stained by Oregon Green, were detected by FISH (green circles). These results suggest that FISH-based viability evaluation does not accurately represent the live/dead status of individual cells.

Cells of anaerobic benzene-degrading bacterium, *Azoarcus* sp. strain DN11 (20), mixed with *E. coli* DH5α cells were analyzed with our method. Approximately 1.0 x 10^7 of DN11 and 0.5 x 10^7 of DH5α were mixed in water, and collected on a membrane filter. After viability check, identification of DN11 cells was performed with *Azoarcus*-specific probe, Alexa546-labeled probe AZ-DN11 (5'

-GCAAGCCTCTCCATTGAGTGA, Invitrogen, Carlsbad, CA). *E. coli* cells was not labeled with the probe. We found both live and dead cells of DN11 could be labeled with FISH, but the probe binding efficiency of live cells（95.3±2.0%) is higher than dead cells (75.8±5.3%). These data suggested that our method is applicable for the viability analysis and specific identification of target bacteria in mixed culture.

As a conclusion, the unique method presented here enables to identify and monitor physiological status of individual target bacterial cells on the same membrane. The key is the usage of glue which can immobilize the cells on membrane filters without affecting their viability and microscopic observation. Together with improved FISH procedure, we could label most of cells with high efficiency. The combined technique can be used for identification and monitoring of viability of targeted bacteria in complex communities.

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249 Fig.1. Sections of membrane filter were adhered to the eight-well printed microscope
250 slide glass with low fluorescent-glue.
251
252
253 Fig.2. (A) Epifluorescence microscope image of *E. coli* DH5α of
254 mid-logarithmic phase, stained with the live/dead fluorescent dyes. Flow-check
255 fluorosphere was used, and located in the same position (upper-left). Oregon
256 green-stained live cells show green color. Intermediate colors like yellow or
257 orange (stained with both of Oregon Green and PI, indicated with arrows) were
258 observed, which we assumed as dying cells. (B) FISH using Cy3-labeled EUB
259 338 probe. Bar, 20 μ m.
260
261
262 Fig.3. Fluorescence images of *P. putida* stain KT2440 (pCAR1::rfp) of late
263 stationary phase stained with Oregon Green (A), and a result of FISH with
264 Cy3-labeled PSU probe (B). Yellow arrows indicate a few live cells showing no
265 RFP signal but stained with Oregon Green in (A) could be detected by FISH
266 signals in (B). Green circles indicate dead cells, which do not emit RFP signal
267 and are not stained with Oregon Green, but detected in FISH experiment. Bar,
268 20 μ m.
269

5. Figure 1

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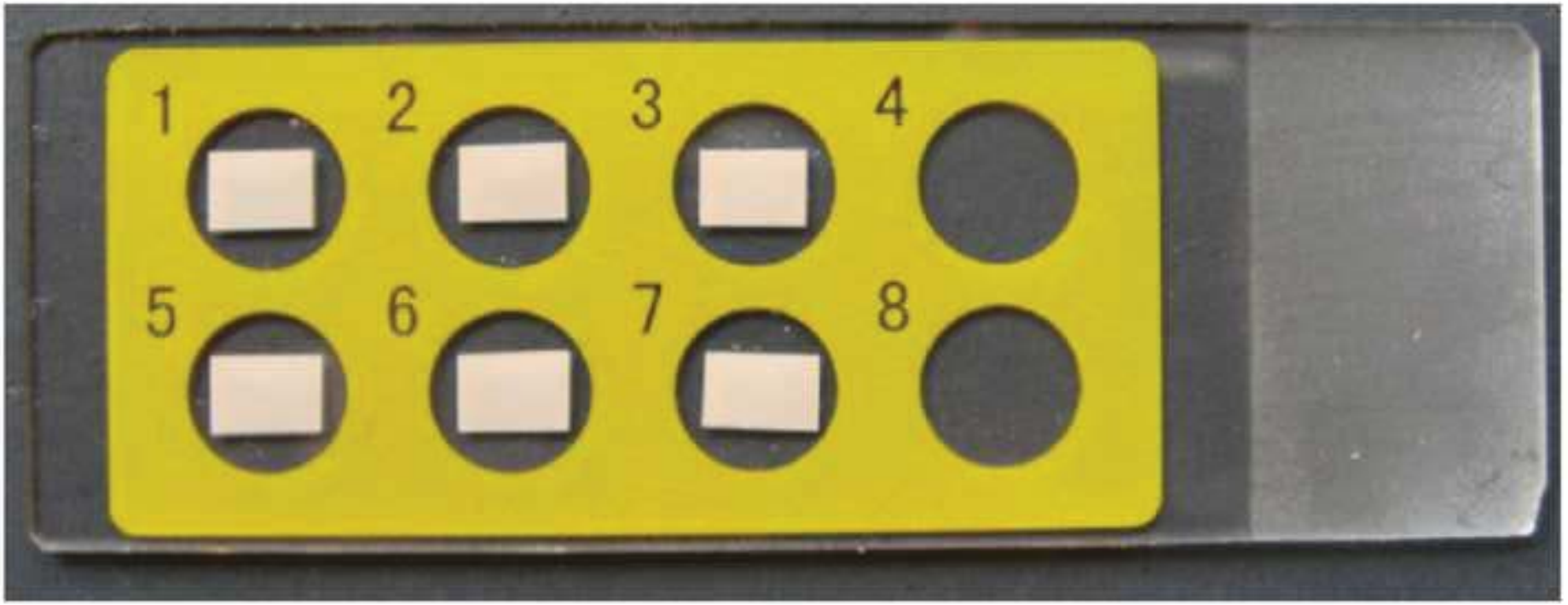

5. Figure 2

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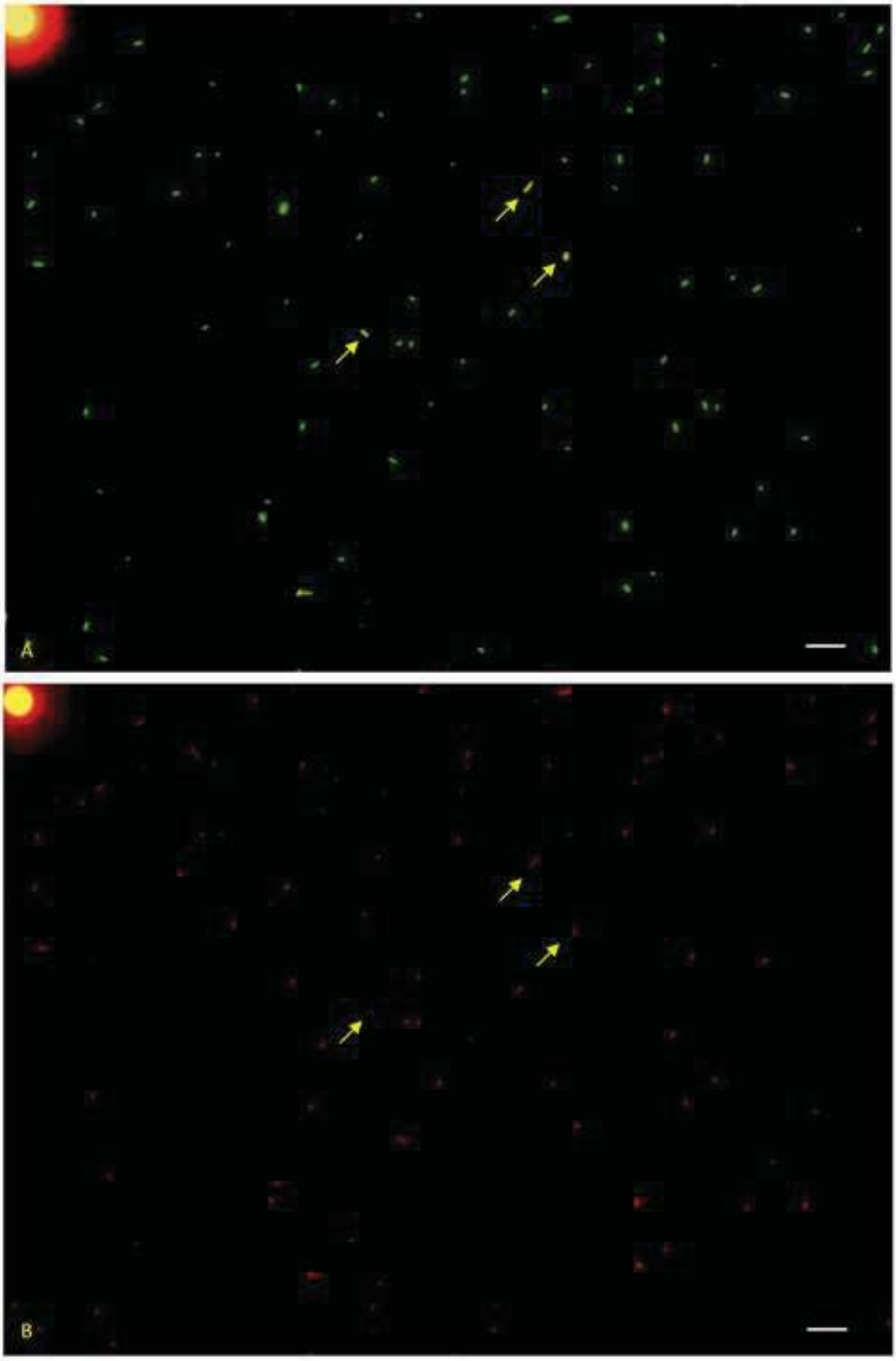

5. Figure 3

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