Rapid Identification and Enumeration of Antibiotic Resistant Bacteria in Urban Canals by Microcolony-Fluorescence in Situ Hybridization

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The abundance and phylogenetic composition of antibiotic resistant bacteria in canals of metropolitan Bangkok, Thailand, were investigated using a microcolony method and fluorescence in situ hybridization (FISH). Cells were directly trapped from aquatic samples onto polycarbonate membranes and incubated for 24 hr on selective agar containing antibiotics. Individual antibiotic resistant bacterial microcolonies samples were classified on the filter using FISH with rRNA-targeted probes. The numbers of microcolony forming units (mCFU) on selective medium containing antibiotics were 0.5 to 8.1-fold (average, 3.4-fold) higher than those of colony forming units (CFU) in all samples, and mCFU and CFU closely correlated in all samples (r² = 0.89). Estimates of Escherichia coli (E. coli) determined by FISH with rRNA-targeted probe accounted for approximately 1% of bacteria detectable by probe EUB338 among microcolony-forming bacteria on nonselective medium. However, they accounted for approximately 10% of bacteria detectable by probe EUB338 among microcolony-forming norfloxacin/tetracycline-resistant bacteria. Microcolony-FISH on selective medium containing antibiotics would be a valuable tool that could help in obtaining information about the numbers and phylogenetic affiliations of yet-to-be-cultured antibiotic-resistant bacteria in aquatic environments.

Key words — microcolony, fluorescence in situ hybridization, antibiotic resistance, river water

INTRODUCTION

Microscope-based approaches with fluorescently labeled probes have become popular tools to gather information on the numbers, phylogenetic affiliations, functions and activities of microorganisms within individual ecosystems without cultivation. Bacteria with physiological activity or specific functions in complex microbial communities have been phylogenetically identified by various methods, including fluorescence in situ hybridization (FISH) using rRNA-targeted oligonucleotide probes combined microautography, direct viable counting, CTC staining, BOBO-3 staining, and the microcolony method.

The microcolony procedure is based on microscopic observation of the early stage of colony formation on laboratory media. The current basic protocol was developed by trapping bacteria on a membrane that is subsequently placed on the surface of a growth medium. Although the relationship between microcolony and plate counts has been determined on heterotrophic medium, quantitative differences remain unclear between microcolonies and colonies on selective medium containing antibiotics and the community composition of antibiotic-resistant microcolonies in aquatic environments. Antibiotic-resistant bacteria and antibiotics are discharged in the environment as a result of the increasing use of antibiotics in medical, veterinary, and agricultural practices. The current basic protocol was developed by trapping bacteria on a membrane that is subsequently placed on the surface of a growth medium. Although the relationship between microcolony and plate counts has been determined on heterotrophic medium, quantitative differences remain unclear between microcolonies and colonies on selective medium containing antibiotics and the community composition of antibiotic-resistant microcolonies in aquatic environments. Antibiotic-resistant bacteria and antibiotics are discharged in the environment as a result of the increasing use of antibiotics in medical, veterinary, and agricultural practices.
ricultural practices. River and canal waters are the main receptacle for these pollutants, since they receive sewage from urban effluent and agricultural wastes. In this study, we combined the microcolony method with FISH to analyze communities of microcolony-forming, antibiotic-resistant bacteria in fresh water environments. Water samples were filtered and incubated on selective agar containing antibiotics and then FISH with rRNA-targeted probes detected individual microcolonies of indigenous bacteria on polycarbonate membranes.

**MATERIALS AND METHODS**

**Sampling Sites** —— Water samples were collected from the Chao Phraya River and the Lord Canal in from January to March (cool season) in 2001 (Fig. 1). Average monthly rainfall is 10 to 30 mm in the cool season and less compared to the rest season. Some sewage plants have been constructed in Bangkok, but the total number is not yet sufficient, and drainpipes are sometimes incomplete. The water quality of the Chao Phraya River around these sites has been monitored by the Pollution Control Department of Thailand. The river water in CP4 and CP5 is medium-clean, fresh water resources used for agriculture. The river water in CP2-1 is fairly clean, fresh water resources used for industrial use. The quality of the Lord Canal is more polluted than the Chao Phraya River.

**Total Direct Counting and Quantitative Direct Counting** —— Total direct counts (TDC) in the river and canal water samples were determined using 4′,6′-diamidino-2-phenylindole (DAPI) at a final concentration of 1 µg ml–1. Viable bacteria in the water samples were determined by quantitative direct viable counting as described by Yokomaku et al. (quantitative direct viable counts, qDVC). This method is based on the selective lysis of viable cells by spheroplast formation after incubating samples with antibiotics, nutrients (yeast extract) and glycine. Antibiotics specifically inhibit DNA synthesis and prevent cell division without affecting other cellular metabolic activities. Glycine induces spheroplast formation by viable cells because glycine interferes with several steps in peptidoglycan synthesis for bacterial cell wall formation. This effect leads to swollen cells with very loose cell walls. The viable cells are lysed by freeze-thaw treatment following spheroplast formation. The number of viable cells was obtained by subtracting the number of remaining cells after the qDVC procedure from the total cell number before the qDVC incubation.

**Plate Counting and Fecal Coliform** —— The number of colony forming units (CFU) was determined by spreading dilute water samples on R2A medium,14) which were incubated at 30°C for 1 week before counting. For enumeration of antibiotic resistant bacteria, 10 or 50 µg ml–1 of ampicillin, 5 or 30 µg ml–1 of norfloxacin, 10 or 50 µg ml–1 of tetracycline was added to R2A agar medium. Fecal coliforms were determined using the MPN method with EC broth.15)

**Microcolony** —— One ml of diluted sample was filtered through 25-mm-diameter polycarbonate membrane filters with pore size of 0.2 µm (ADVANTEC Co., Tokyo, Japan). Membrane filters were transferred to petri dishes containing 10-fold diluted R2A agar medium and incubated at 30°C for 24 hr. For enumeration of microcolony forming antibiotic resistant bacteria, 10 or 50 µg ml–1 of ampicillin, 5 or 30 µg ml–1 of norfloxacin, 10 or 50 µg ml–1 of tetracycline was added to 10-fold diluted R2A agar medium. Microcolony forming units (mCFU) were obtained after the 24 hr-incubation. To examine the community composition of microcolony forming bacteria, FISH analysis was carried out as described below.

**Oligonucleotide Probes** —— The following oligonucleotide probes were used: EUB338, complementary to a region of the 16S rRNA conserved in the domain bacteria;16) NON338, negative control;17) CF319, complementary to a region of the 16S rRNA specific Cytophaga-Flavobacterium phylum;18) ALF1b, complementary to a region of the 16S rRNA specific the alpha subclass of Proteobacteria;19)
BET42a, complementary to a region of the 23S rRNA specific for the beta subclass of Proteobacteria; ES445, complementary to a region of the 23S rRNA specific for the gamma subclass of Proteobacteria; and GAM42a, complementary to a region of the 16S rRNA specific for Escherichia coli (E. coli). Probes were labeled with CY3 at 5’ end.

**Fluorescence in Situ Hybridization** — 12% (w/v) paraformaldehyde in phosphate-buffered saline [PBS; 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na₂PO₄, 1.5 mM KH₂PO₄ (pH 7.2)] was added to the collected samples in a final concentration of 3% (w/v) and cells were fixed at 4°C overnight. Cells were concentrated from water samples on a black polycarbonate filter (diameter, 25 mm; pore size, 0.2 µm; ADVANTEC). Hybridization on filters was performed referring Glöckner et al.²⁰ For EUB338, NON338, CF319, ALF1b, BET42a, and GAM42a probes, hybridization and washing conditions were carried out as described by Glöckner et al.²⁰ For ES445 probe, the protocol was modified from that described by Kenzaka et al.²¹ Hybridization was performed in a moisture chamber at 41°C for 2 hr with hybridization buffer [0.45 M NaCl, 20 mM Tris-HCl (pH 8.0), 0.1% sodium dodecyl sulfate (SDS)] containing 2 ng µl⁻¹ of probe. The washing step was done at 41°C for 30 min with washing buffer [0.08 M NaCl, 20 mM Tris-HCl (pH 8.0), 0.1% SDS]. Counterstaining was carried out with 2 µg ml⁻¹ of DAPI.

**Microcolony-Fluorescence in Situ Hybridization** — Polycarbonate membranes incubated for microcolony formation were placed on filter paper soaked with 3% (w/v) paraformaldehyde in PBS and microcolonies were fixed at 4°C overnight. Polyacrylamide gel was embedded to maintain fixed microcolonies in the 3-dimensional native hydrated state and to simultaneously permit easy handling of the fixed microcolony. The embedding solution consisted of 250 µl of 20% acrylamide-bisacrylamide) mixed with 2 µl of N,N,N',N'-tetramethylethylenediamine (TEMED). Immediately before inoculation, 5 µl of ammonium persulfate (APS) was added and 10 µl of the mixture was spotted onto glass slides and polycarbonate membranes with microcolonies were placed on the solution. The mixture diffused under the membrane and solidified at room temperature (approximately 25°C) for 10 min. Thereafter, 50 µl of fresh acrylamide containing TEMED and APS as described above was spotted onto the membranes and coverslip (16 × 16 mm) was placed on top. The acrylamide solidified at room temperature for 10 min. The coverslip was peeled off and the polyacrylamide-coated membrane was cut into 4 pieces. Hybridization proceeded on the membranes as described above.

**Microscopy** — The filter sections were observed with ECLIPSE E400 epifluorescence microscope (Nikon, Tokyo, Japan) equipped with a 100-W mercury burner and specific filter sets (UV-2A for DAPI; HQ:CY3 for CY3). For FISH, more than 1500 DAPI-stained cells were counted per sample. For microcolony-FISH, more than 200 DAPI-stained microcolonies were counted per sample. All counts were corrected by subtracting the counts obtained with the negative control probe NON338.

### RESULTS

**Microbial Characteristics of River and Canal Waters**

The number of total bacteria, viable bacteria, colony forming bacteria, E. coli and fecal coliforms in river and canal water samples was shown in Table 1. Generally, the numbers of bacteria increased with the level of water pollution. Table 2 shows the correlation coefficient of fecal and antibiotic resistant bacteria in river and canal samples (n = 15).

| Table 1. Microbial Characteristics of the Sampling Sites²¹ |  |
|---|---|---|---|---|---|---|
| Site | TDC (10⁶ ml⁻¹) | qDVC (10⁶ ml⁻¹) | CFU (10⁷ ml⁻¹) | ES445 (10⁴ ml⁻¹) | FC (10⁴ ml⁻¹) |  |
| CP5 | 1.9 ± 0.65 | 1.1 ± 0.58 | 0.91 ± 0.60 | 6.6 ± 7.9 | 4.9 |  |
| CP4 | 1.6 ± 0.68 | 1.1 ± 0.18 | 6.4 ± 2.5 | 0.85 ± 1.3 | 2.4 |  |
| CP2-1 | 2.5 ± 0.78 | 1.5 ± 0.28 | 5.4 ± 1.1 | 5.1 ± 3.5 | 9.5 |  |
| L2 | 2.8 ± 1.1 | 2.3 ± 0.23 | 5.0 ± 1.4 | 9.3 ± 3.2 | 110 |  |
| L3 | 5.9 ± 1.2 | 4.7 ± 0.51 | 31 ± 23 | 16 ± 7.3 | 350 |  |

*a* TDC, total direct counts; qDVC, quantitative direct viable counts; CFU, colony forming units; ES445, E. coli counts determined with fluorescence in situ hybridization; FC, fecal coliforms determined with MPN method. Mean and standard deviations of triplicate samples were shown.
The abundance of antibiotic bacteria closely correlated with that of fecal coliforms and of *E. coli* determined by FISH.

**Microcolony-Forming, Antibiotic-Resistant Bacteria**

The optimal concentration of R2A medium for microcolony formation was examined among the indigenous bacteria in canal samples. R2A medium without dilution allowed the rapid growth and formation of large microcolonies of certain populations, which hindered the observation of smaller microcolonies. Growth was obviously retarded when R2A was diluted 1000-fold, but dilutions of 10- and 100-fold did not affect either the growth rate or the numbers of microcolonies. We established the optimal R2A dilution at 10-fold, and also used this dilution to determine the numbers of microcolonies that formed antibiotic-resistant bacteria.

The numbers of microcolonies that formed antibiotic- (ampicillin, norfloxacin, tetracycline) resistant bacteria in canal water taken from site L3 was compared with those obtained by conventional plate counting in Fig. 2A, and the relationships in antibiotics with higher concentration (50 µg ml⁻¹ of ampicillin, 30 µg ml⁻¹ of norfloxacin, 50 µg ml⁻¹ of tetracycline) at all sites were summarized in Fig. 2B. The ratios of mCFU to CFU ranged from 0.5 to 8.1 (mean, 3.7), and mCFU and CFU closely correlated in the water samples ($r^2 = 0.89$, $p < 0.01$).

**Composition of Microcolony-Forming Bacteria**

Polyacrylamide gel was embedded after microcolony formation to maintain the structure of...
microcolonies for FISH. Although polyacrylamide slightly obscured the fluorescence of microcolonies hybridized with the CY3-labeled probe, microcolonies hybridized with the rRNA-targeted probe were easily counted using an epifluorescence microscope. Representative photomicrographs of microcolonies in canal water samples detected by FISH with probe EUB338 and BET42a are shown in Fig. 3. To exclude the possibility of nonspecific probe binding to polyacrylamide gel or to microcolony structures other than target rRNA in the bacterial cells, we also hybridized the NON338 probe. Microcolony fluorescence was insignificant in the presence of this probe.

We examined the phylogenetic composition of antibiotic-resistant bacteria in canal water taken from site L3 using the microcolony method combined with FISH. We classified the bacterial community using oligonucleotide probes specific for domain bacteria (EUB338) and for five bacterial groups (Flavobacterium-Cytophaga; the alpha, beta and gamma subclasses of Proteobacteria; E. coli) (Fig. 4). 78% of DAPI-stained cells in the resident bacterial community without incubation for microcolonies was detected by FISH with probe EUB338 (Fig. 4, “resident”).

Bacteria in canal water sample frequently hybridized with probe BET42a. The beta subclass of Proteobacteria constituted a dominant fraction among the microcolony-forming bacteria on nonselective medium and on selective medium containing antibiotics. The label “Not identified” refers to the share of bacteria detectable by the probe EUB338 that could not be explained by the sum of bacteria detectable with the five group-specific probes. The “Not identified” population accounted for about 10% of the DAPI stained cells among the resident bacteria that were not incubated for microcolony formation, but the population constituted a large fraction among the microcolony-forming bacteria incubated on 10-fold diluted R2A medium and among the ampicillin- or norfloxacin-resistant bacteria. The E. coli group accounted for about 1% of the bacteria detected by probe EUB338 among both the resident bacterial community without incubation for microcolonies and microcolony-forming bacteria. However, they accounted for about 10% of the bacteria detected by probe EUB338 among the

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Fig. 3. Micrographs Typical of Microcolony-Forming, Antibiotic-Resistant Bacteria Detected by Fluorescence in Situ Hybridization
(A) Norfloxacin-resistant microcolony from bacteria found in the Lord Canal hybridized with Cy3-labeled EUB338 probe. Only hybridized bacteria emitted orange Cy3 fluorescence under green excitation. (B) Same microscopic fields as on the left with UV excitation. (C) Tetracycline-resistant microcolony from bacteria found in the Lord Canal hybridized with Cy3-labeled Beta42a probe. Only hybridized bacteria emitted orange Cy3 fluorescence under green excitation. (D) Same microscopic fields as on the left with UV excitation.
microcolony-forming bacteria that were resistant to norfloxacin/tetracycline.

**DISCUSSION**

Plate counting techniques using selective medium containing antibiotics play an important role in studying the incidence, distribution of antibiotic resistant bacteria and the gene types of the isolates in natural environments. However, they might underestimate the actual antibiotic resistant population because a considerable fraction of bacterial communities in the natural environment does not form visible colonies on established nonselective media. To address the question, we examined quantitative differences between microcolonies and colonies on selective medium containing antibiotics in urban canal waters.

The comparison of plate and microcolony counts on selective medium showed that the quantitative differences were not over orders of magnitude in the urban river and canal, but the numbers of antibiotic-resistant bacteria determined by these methods significantly differed (Fig. 2). Correlation between mCFU and CFU was found in the water samples. Since the microcolony method can recognize the early stages of colony formation on laboratory medium using an epifluorescence microscope, it is more rapid than conventional plate counting. Incubation for 2 to 7 days is often required for formation of visible colonies, whereas the microcolony method can generate results in one day.

Most studies using rRNA-targeted in situ hybridization have demonstrated that the beta subclass of Proteobacteria constitutes the dominant fraction in freshwater systems. This was also observed in the present study (Fig. 4). The gamma subclass is thought to constitute only a small fraction in various fresh water systems. Our results of microcolony-FISH on water samples plated on 10-fold diluted R2A medium showed that the gamma Proteobacteria constituted 21% of the EUB338 counts, although it represented only 5.3% of the resident bacterial community without incubation for microcolony formation. Many members of this group adapt to high nutrient concentrations, and therefore grow well under laboratory conditions. Our results also showed that the Proteobacteria responded well to nutrients, which agrees with the findings from Thai river water in a previous study.

The level of *E. coli* contamination in fresh water is the mean of estimating the degree of recent fecal pollution in most countries. We used the ES445
probe that is specific for E. coli, in the FISH assay. The abundance of E. coli determined by FISH among microcolony-forming, antibiotic-resistant bacteria was significantly higher (p < 0.01) than that among resident bacteria without incubation for microcolony formation or among microcolony-forming bacteria on nonselective medium (Fig. 4). The abundance of E. coli determined by FISH closely correlated (r² = 0.99, p < 0.05) with that of fecal coliforms determined using the MPN method with EC broth as well as that of antibiotic-resistant bacteria in river and canal water samples (Table 2). Many studies on antibiotic resistance in the aquatic habitat have concerned bacteria of fecal origin. In addition, urban discharge resulted in the increase of the rates of antibiotic-resistant bacteria in river. The microcolony-FISH using appropriate probes and antibiotics should be a useful tool for assessing threats to public health from contaminated waters.

The microcolony concept has been recently re-evaluated and the growth of microorganisms in a simulated natural environment or an environment with limited nutrients has enabled microcolony formation of bacteria that have been resistant to cultivation and microautoradiography. Thus microcolony-FISH on selective medium (Fig. 4). The abundance of E. coli determined by FISH closely correlated (r² = 0.99, p < 0.05) with that of fecal coliforms determined using the MPN method with EC broth as well as that of antibiotic-resistant bacteria in river and canal water samples (Table 2). Many studies on antibiotic resistance in the aquatic habitat have concerned bacteria of fecal origin. In addition, urban discharge resulted in the increase of the rates of antibiotic-resistant bacteria in river. The microcolony-FISH using appropriate probes and antibiotics should be a useful tool for assessing threats to public health from contaminated waters.

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