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

Takehiko Kenzaka,[a, b] Nobuyasu Yamaguchi,[b] Fuangfa Utrarachkij,[c] Orasa Suthienkul,[c] and Masao Nasu*[, b]

[a]*Faculty of Environment and Resource Studies, Mahidol University, 4, Phuttamonthon, Salaya, Nakorn Pathom, 73170, Thailand,* [b]*Graduate School of Pharmaceutical Sciences, Osaka University, 1–6, Yamada-oka, Suita, Osaka 565–0871, Japan, and* [c]*Faculty of Public Health, Mahidol University, 420/1, Rajvithi Rd., Bangkok, 10400, Thailand*

(Received June 10, 2006; Accepted September 12, 2006)

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 ($r^2 = 0.89$). 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,[1] direct viable counting,[2] CTC staining,[3] BOBO-3 staining,[4] and the microcolony method.[5]

The microcolony procedure is based on microscopic observation of the early stage of colony formation on laboratory media.[6] The current basic protocol was developed by trapping bacteria on a membrane that is subsequently placed on the surface of a growth medium.[7] The microcolony method with specific medium can selectively enumerate target species. Specific substrates in combination with the microcolony method allow direct visualization of microorganisms with metabolic potential within a complex community.[7]

The microcolony method with selective medium containing antibiotics would detect antibiotic-resistant bacteria more rapidly and accurately than conventional plating methods. Although the relationship between microcolony and plate counts has been determined on heterotrophic medium,[8–10] 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 ag-

*To whom correspondence should be addressed: Graduate School of Pharmaceutical Sciences, Osaka University, 1–6, Yamada-oka, Suita, Osaka 565–0871, Japan. Tel.: +81-6-6879-8170; Fax: +81-6-6879-8174; E-mail: nasu@phs.osaka-u.ac.jp

ricultural practices.[11] 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.[12] 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).[13] 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.

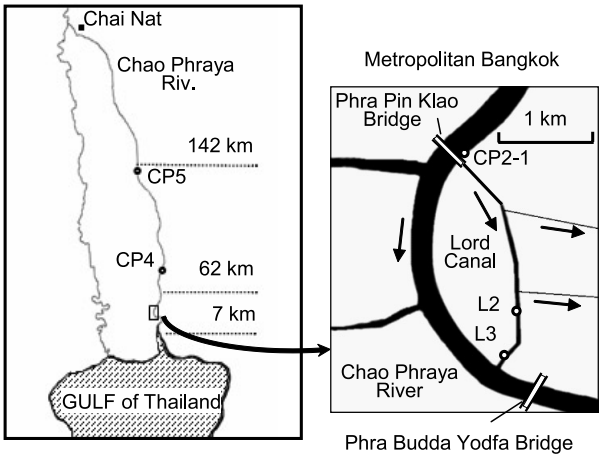

Fig. 1. Sampling Sites in the Chao Phraya River and the Lord Canal, Thailand

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*;[19] GAM42a, complementary to a region of the 23S rRNA specific for the gamma subclass of *Proteobacteria*;[19] ES445, complementary to a region of the 16S rRNA specific for *Escherichia coli* (*E. coli*).[2] 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 Na_2PO_4, 1.5 mM KH_2PO_4 (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*.[2] 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 (200 : 1 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 Sites[a]

Site	TDC (10^6 ml^{-1})	qDVC (10^6 ml^{-1})	CFU (10^4 m^{-1})	ES445 (10^4 ml^{-1})	FC (10^1 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.

Table 2. Correlation Half-Matrix of Microbial Parameter[a)]

Parameter	Correlation coefficient for:							
	ES445	FC	Amp10	Amp50	Nor5	Nor30	Tet10	Tet50
ES445	1.00							
FC	0.92	1.00						
Amp10	0.85	0.96	1.00					
Amp50	0.86	0.96	1.00	1.00				
Nor5	0.71	0.92	0.96	0.96	1.00			
Nor30	0.85	0.96	1.00	1.00	0.96	1.00		
Tet10	0.88	0.97	0.99	1.00	0.94	0.99	1.00	
Tet50	0.89	0.98	1.00	1.00	0.95	0.99	0.99	1.00

a) ES445, *E. coli* counts determined with fluorescence in situ hybridization; FC, fecal coliforms; Amp10, Amp50, ampicillin-resistant bacteria (concentrations of ampicillin was 10 or 50 μg ml^{-1}, respectively); Nor5, Nor30, norfloxacin-resistant bacteria (concentrations of norfloxacin was 5 or 30 μg ml^{-1}, respectively); Tet10, Tet50, tetracycline-resistant bacteria (concentrations of tetracycline was 10 or 50 μg ml^{-1}, respectively) ($n = 15$).

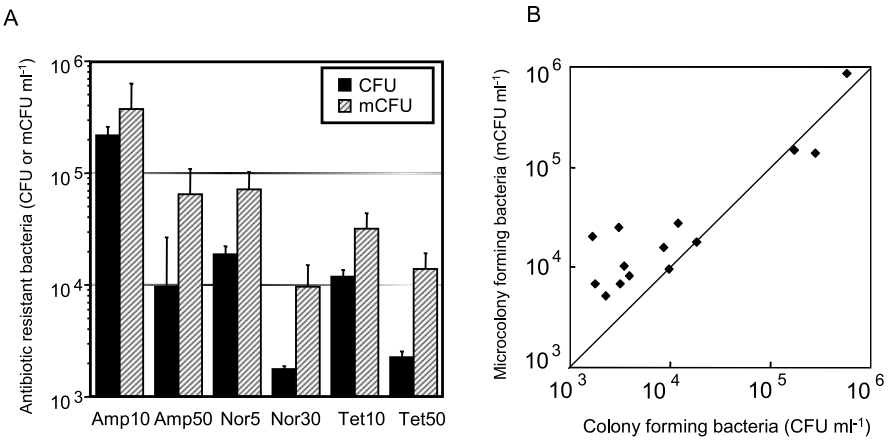

Fig. 2. Comparison of Number of Antibiotic Resistant Bacteria in Canal Water Samples from L3 (A) and All Sites (B) Determined by CFU on R2A Medium Containing Antibiotics and mCFU on 10-Fold Diluted R2A Medium Containing Antibiotics

Bars represent standard deviations. Amp10 and Amp50, bacteria resistant to 10 and 50 μg ml^{-1} ampicillin respectively; Nor5 and Nor30, bacteria resistant to 5 and 30 μg ml^{-1} norfloxacin, respectively; Tet10 and Tet50, bacteria resistant to 10 and 50 μg ml^{-1} tetracycline, respectively.

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

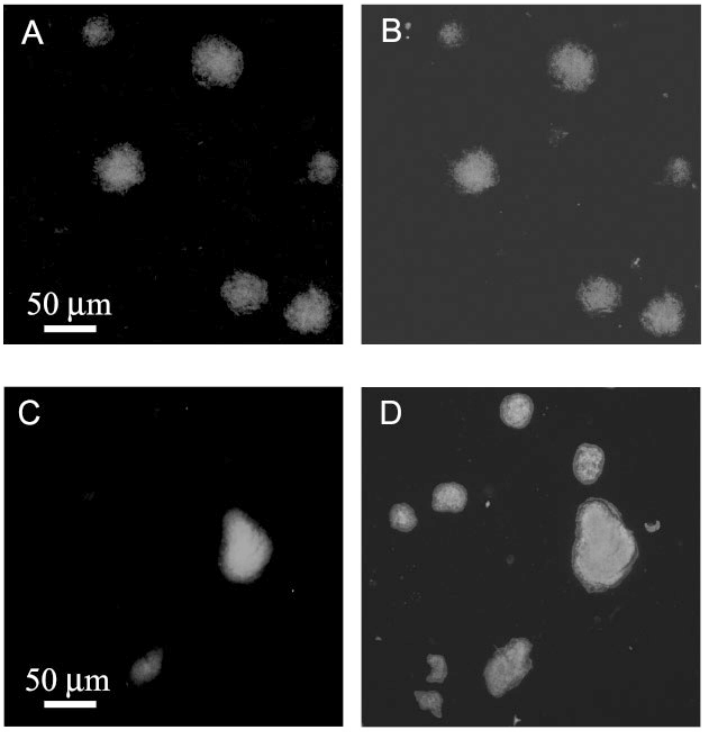

Fig. 3. Micrographs Typical of Microcolony-Forming, Antibiotic-Resistant Bacteria Detected by Fluorescence *in Situ* Hybridization

(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.

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

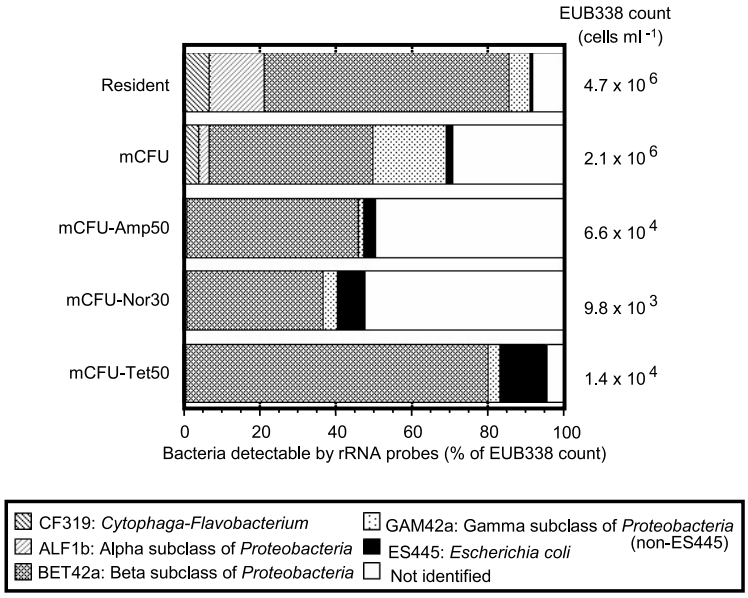

Fig. 4. Composition of Antibiotic-Resistant Bacteria in Canal Water Samples from L3 Determined by Fluorescence *in Situ* Hybridization Combined with Microcolony Method

Resident, resident bacterial community without incubation for microcolony formation; mCFU, microcolony forming bacteria growing on 10-fold diluted R2A agar without antibiotics; mCFU-Amp50, microcolony-forming, ampicillin-resistant bacteria growing on 10-fold diluted R2A agar with 50 μg ml^{-1} of ampicillin; mCFU-Nor30, microcolony-forming, norfloxacin-resistant bacteria growing on 10-fold diluted R2A agar medium with 30 μg ml^{-1} of norfloxacin; mCFU-Tet50, microcolony forming tetracycline-resistant bacteria growing on 10-fold diluted R2A agar with 50 μg ml^{-1} of tetracycline.

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.[11,21,22] 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.[23] 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.[2,20,24] 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.[25] Our results also showed that the *Proteobacteria* responded well to nutrients, which agrees with the findings from Thai river water in a previous study.[2]

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 ($r^2 = 0.99$, $p < 0.05$) 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.[11] In addition, urban discharge resulted in the increase of the rates of antibiotic resistant bacteria in river.[21] 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 culture on standard media.[26–28] Most marine or soil bacteria under environmental conditions grow to the microcolony stage.[26,29] Microcultivation combined with FISH allows the identification of previously "unculturable" bacteria by fluorescence microscopy.[30,31] Thus microcolony-FISH on selective medium containing antibiotics would be a valuable tool with which to rapidly gain information on the number and phylogenetic affiliation of so far uncultured, antibiotic-resistant bacteria from aquatic environments.

Acknowledgements This study was supported by the JSPS Grant-in-Aid for Scientific Research (A) (17256001) and JSPS Grant-in-Aid for Scientific Research (B) (11691152).

REFERENCES

1) Lee, N., Nielsen, P. H., Andreasen, K. H., Juretschko, S., Nielsen, J. L., Schleifer, K. H. and Wagner, M. (1999) Combination of fluorescent in situ hybridization and microautoradiography — a new tool for structure-function analyses in microbial ecology. *Appl. Environ. Microbiol.*, **65**, 1289–1297.
2) Kenzaka, T., Yamaguchi, N., Prapagdee, B., Mikami, E. and Nasu, M. (2001) Bacterial community composition and activity in urban rivers in Thailand and Malaysia. *J. Health Sci.*, **47**, 353–361.
3) Kitaguchi, A., Yamaguchi, N. and Nasu, M. (2005) Enumeration of respiring Pseudomonas spp. in milk within 6 hours by fluorescence in situ hybridization following formazan reduction. *Appl. Environ. Microbiol.*, **71**, 2748–2752.
4) Kitaguchi, A., Yamaguchi, N. and Nasu, M. (2006) Simultaneous enumeration of viable Enterobacteriaceae and *Pseudomonas* spp. within three hours by multicolor fluorescence in situ hybridization with vital staining. *J. Microbiol. Methods*, **65**, 623–627.
5) Stender, H., Oliveira, K., Rigby, S., Bargoot, F. and Coull, J. (2001) Rapid detection, identification, and enumeration of *Escherichia coli* by fluorescence in situ hybridization using an array scanner. *J. Microbiol. Methods*, **45**, 31–39.
6) Torella, F. and Morita, R. Y. (1981) Microcultural study of bacterial size changes and microcolony and ultramicrocolony formation by heterotrophic bacteria in seawater. *Appl. Environ. Microbiol.*, **41**, 518–527.
7) Rodrigues, U. M. and Kroll, R. G. (1988) Rapid selective enumeration of bacteria in foods using a microcolony epifluorescence microscopy technique. *J. Appl. Bacteriol.*, **64**, 65–78.
8) Rodrigues, U. M. and Kroll, R. G. (1989) Microcolony epifluorescence microscopy for selective enumeration of injured bacteria in frozen and heat-treated foods. *Appl. Environ. Microbiol.*, **55**, 778–787.
9) Kawai, M., Yamaguchi, N. and Nasu, M. (1999) Rapid enumeration of physiologically active bacteria in purified water used in the pharmaceutical manufacturing process. *J. Appl. Microbiol.*, **86**, 496–504.
10) Hesselsoe, M., Brandt, K. K. and Sørensen, J. (2001) Quantification of viable ammonia-oxidizing bacteria in soil using microcolony technique combined with fluorescent in situ hybridization (MCFU-FISH). *FEMS Microbiol. Ecol.*, **38**, 87–95.
11) Kümmerer, K. (2004) Resistance in the environment. *J. Antimicrob. Chemother.*, **54**, 311–320.
12) Pollution Control Department, Ministry of Science, Technology and Environment (1995) *Pollution Thailand 1995*.
13) Yokomaku, D., Yamaguchi, N. and Nasu, M. (2000) Improved direct viable count procedure for quantitative estimation of bacterial viability in freshwater environments. *Appl. Environ. Microbiol.*, **66**, 5544–5548.
14) Reasoner, D. J. and Gerldreich, E. E. (1985) A new medium for the enumeration and subculture of bacteria from potable water. *Appl. Environ. Microbiol.*, **49**, 1–7.

15) Hurst, C. J., Knudsen, G. R., McInerney, M. J., Stetzenbach, L. D. and Walter, M. V. (1997) *Manual of environmental microbiology*, American Society for Microbiology, Washington, D.C., pp. 187–189.
16) Amann, R. I., Binder, B. J., Olson, R. J., Chisholm, S. W., Devereux, R. and Stahl, D. A. (1990) Combination of 16S rRNA-targeted oligonucleotide probes with flow cytometry for analyzing mixed microbial populations. *Appl. Environ. Microbiol.*, **56**, 1919–1925.
17) Wallner, G., Amann, R. and Beisker, W. (1993) Optimizing fluorescent in situ hybridization with rRNA-targeted oligonucleotide probes for flow cytometric identification of microorganisms. *Cytometry*, **14**, 136–143.
18) Manz, W., Amann, R., Ludwig, W., Vancanneyt, M. and Schleifer, K. H. (1996) Application of a suite of 16S rRNA-specific oligonucleotide probes designed to investigate bacteria of the phylum *Cytophaga-Flavobacter-Bacteroides* in the natural environment. *Microbiology (Reading, U. K.)*, **142**, 1097–1106.
19) Manz, W., Amann, R., Ludwig, W., Wagner, M. and Schleifer, K. H. (1992) Phylogenetic oligodeoxynucleotide probes for the major subclasses of Proteobacteria: problems and solutions. *Syst. Appl. Microbiol.*, **15**, 593–600.
20) Glöckner, F. O., Amann, R., Alfreider, A., Pernthaler, J., Psenner, R., Trebesius, K. and Schleifer, K. H. (1996) An in situ hybridization protocol for detection and identification of planktonic bacteria. *Syst. Appl. Microbiol.*, **19**, 403–406.
21) Goni-Urriza, M., Capdepuy, M., Arpin, C., Raymond, N., Caumette, P. and Quentin, C. (2000) Impact of an urban effluent on antibiotic resistance of riverine Enterobacteriaceae and Aeromonas spp. *Appl. Environ. Microbiol.*, **66**, 125–132.
22) Teuber, M. (2001) Veterinary use and antibiotic resistance. *Curr. Opin. Microbiol.*, **4**, 493–499.
23) Byrd, J. J., Xu, H. S. and Colwell, R. R. (1991) Viable but nonculturable bacteria in drinking water. *Appl. Environ. Microbiol.*, **57**, 875–878.
24) Alfreider, A., Pernthaler, J., Amann, R., Sattler, B., Glockner, F., Wille, A. and Psenner, R. (1996) Community analysis of the bacterial assemblages in the winter cover and pelagic layers of a high mountain lake by in situ hybridization. *Appl. Environ. Microbiol.*, **62**, 2138–2144.
25) Wagner, M., Amann, R., Lemmer, H. and Schleifer, K. H. (1993) Probing activated sludge with oligonucleotides specific for proteobacteria: inadequacy of culture dependent methods for describing microbial community structure. *Appl. Environ. Microbiol.*, **59**, 1520–1525.
26) Kaeberlein, T., Lewis, K. and Epstein, S. S. (2002) Isolating 'uncultivable' microorganisms in pure culture in a simulated natural environment. *Science*, **296**, 1127–1129.
27) Zengler, K., Toledo, G., Rappe, M., Elkins, J., Mathur, E. J., Short, J. M. and Keller, M. (2002) Cultivating the uncultured. *Proc. Natl. Acad. Sci. U.S.A.*, **99**, 15681–15686.
28) Simu, K. and Hagstrom, A. (2004) Oligotrophic bacteriplankton with a novel single-cell life strategy. *Appl. Environ. Microbiol.*, **70**, 2445–2451.
29) Winding, A., Binnerup, S. and Sørensen, J. (1994) Viability of indigenous soil bacteria assayed by respiratory activity and growth. *Appl. Environ. Microbiol.*, **60**, 2869–2875.
30) Binnerup, S., Bloem, J., Hansen, B., Wolters, W., Veninga, M. and Hansen, M. (2001) Ribosomal RNA content in microcolony forming soil bacteria measured by quantitative 16S rRNA hybridisation and image analysis. *FEMS Microbiol. Ecol.*, **37**, 231–237.
31) Ferrari, B. C., Binnerup, S. J. and Gillings, M. (2005) Microcolony cultivation on a soil substrate membrane system selects for previously uncultured soil Bacteria. *Appl. Environ. Microbiol.*, **71**, 8714–8720.