rRNA-targeted fluorescent *in situ* hybridization analysis of bacterial community structure in river water

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An improved *in situ* hybridization technique, HNPP-FISH, using 2-hydroxy-3-naphthoic acid 2'-phenylanilide phosphate (HNPP) and Fast Red TR was applied to analyse the community structure of planktonic bacteria in river water. Oligonucleotide probes specific for the domain Bacteria (EUB338) and five bacterial groups [*Flavobacterium*–*Cytophaga*; *Burkholderia*–*Pseudomonas* (rRNA III)–authentic *Alcaligenes*; *Vibrio*–*Aeromonas*; *Pseudomonas* (rRNA I); the genus *Acinetobacter*] were used to investigate the bacterial community structure at two sites differing in organic carbon pollution level. At the eutrophic site, 54–68% of all cells visualized by staining with DAPI (4',6-diamidino-2-phenylindole) could be detected with probe EUB338. In samples from the oligotrophic site, 39–45% of the total cells hybridized with EUB338. At the eutrophic site, approximately 50% of the total cells were identified with the five group-specific probes; the bacterial community structure was dominated by the *Flavobacterium*–*Cytophaga* group and *Burkholderia*–*Pseudomonas* (rRNA III)–authentic *Alcaligenes* group. At the oligotrophic site, only 26–38% of the total cells were identified with the five group-specific probes. The community structure at the oligotrophic site was similar to that at the eutrophic site, although the percentage of EUB338-detectable cells differed. No appreciable change was found in the community structure during the sampling period at either site. The improved HNPP-FISH technique should be a useful tool for the analysis of microbial community composition.

Keywords: rRNA-targeted oligonucleotide probes, fluorescent *in situ* hybridization, river, bacterial community structure

INTRODUCTION

Complex microbial communities carry out various environmental processes which are essential to maintain ecosystems, and the biogeochemical significance of bacteria is well established (Belser, 1979; Lovley, 1991; Wolin & Miller, 1987). However, current knowledge of the structure and dynamics of natural microbial communities is rather limited, and the bacteria are still treated as a 'black box' in most ecological studies. Most studies on microbial community composition have been based on culture-dependent methods (Maeda, 1980;

Peele *et al.*, 1981; Torsvik *et al.*, 1990) despite the fact that many environmental bacteria cannot yet be cultivated by conventional laboratory techniques (Kogure *et al.*, 1979; Olsen & Bakken, 1987).

Various approaches have been used to identify microorganisms in natural samples without the requirement for laboratory cultivation. These include direct sequence analysis, sequencing of extracted 5S rRNAs, and analysis of rRNA using reverse transcriptase or cloned rRNA genes obtained by amplification using PCR (Giovannoni *et al.*, 1990; Stahl *et al.*, 1985; Weisburg *et al.*, 1991). A new technique, denaturing gradient gel electrophoresis has been developed which identifies sequence variations in a number of genes from complex microbial communities (Teske *et al.*, 1996). However, the extraction, purification and amplification of nucleic acid from

Abbreviations: FISH, fluorescent *in situ* hybridization; HNPP, 2-hydroxy-3-naphthoic acid 2'-phenylanilide phosphate.

original samples is often selective and reductive (e.g. Suzuki & Giovannoni, 1996).

Whole-cell hybridization techniques with fluorescently labelled, rRNA-targeted oligonucleotide probes allow not only detection of specific bacteria at the single-cell level but also determination of their abundance in complex communities (Amann *et al.*, 1990; Hicks *et al.*, 1992; Wallner *et al.*, 1995). Investigations of this type on a natural aquatic system have been carried out by several researchers (Hicks *et al.*, 1992; Glöckner *et al.*, 1996; Ramsing *et al.*, 1996). However, the application of *in situ* hybridization to the oligotrophic environment remains limited (Alfreider *et al.*, 1996). This is primarily because the copy number of rRNA-binding sites in naturally occurring bacteria is much lower than in cultured systems (Amann *et al.*, 1995). Various approaches to amplifying signals on the basis of an enzymic reaction have been developed (Lebaron *et al.*, 1997; Schhuber *et al.*, 1997). We recently reported a new fluorescent *in situ* hybridization technique, HNPP-FISH, using 2-hydroxy-3-naphthoic acid 2'-phenylanilide phosphate (HNPP) and Fast Red TR, which enhances the fluorescent signals eightfold compared to standard fluorescent *in situ* hybridization (FISH) with mono-FITC-labelled oligonucleotide probes (Yamaguchi *et al.*, 1996). In the study reported here, this technique was applied to the analysis of planktonic bacterial cells in river water.

METHODS

Organisms and culture conditions. Growth conditions and media for the organisms used in this study were as follows: *Escherichia coli*, 37 °C in LB medium (1% tryptone, 0.5% yeast extract, 0.5% NaCl; pH 7.0); all other bacterial species, 30 °C in LB medium (genus *Flavobacterium*, 0% NaCl; genus *Vibrio*, 3% NaCl; all other strains, 0.5% NaCl) (Yamaguchi *et al.*, 1996).

Sampling. River water was collected at Takayama (34° 52' 12" N, 135° 28' 60" E) and Kitahashi (34° 41' 27" N, 135° 32' 3" E) in northern part of Osaka, Japan (Fig. 1). Characteristics of the river water are listed in Table 1. Takayama is surrounded by forest. At this point, the river is narrow, shallow and fast-flowing. The stream bed is very rocky and the water is not exposed to domestic or industrial effluents. Kitahashi is located in a commercial area, Osaka Business Park, and considered to be highly polluted. Domestic water flows into this river upstream. Water samples were collected from the surface at each site and the physico-chemical and microbiological parameters regularly determined. The microbial community in the river water samples taken in January, April, and October of 1996 was analysed by *in situ* hybridization.

Oligonucleotide probes. An aligned database of 16S and 23S rRNA sequences was obtained from GenBank (Release 84.0). Design of oligonucleotide probes for specific bacterial groups was carried out as described previously (Yamaguchi *et al.*, 1996). Five probes specific for different groups of *Proteobacteria* and the *Flavobacterium*-*Cytophaga* cluster were designed (Fig. 2, Table 2). These probes are targeted to regions of the 16S rRNA characteristic for the *Flavobacterium*-*Cytophaga* group (FC), the *Burkholderia*-*Pseudomonas*

Fig. 1. Map of northern Osaka. The sampling sites are marked by black circles. Osaka University is shown as a black square. The Minoh river originates in the Minoh Mountains and joins the Ina river. Takayama is located upstream of the Minoh river. Kitahashi is located downstream of the Neya river. These rivers both flow into Osaka Bay.

(rRNA III)-authentic *Alcaligenes* group (BPA), the genus *Acinetobacter* (Ac) and the *Vibrio*-*Aeromonas* group (VA), and to regions of 23S rRNA for *Pseudomonas* (rRNA I) [P(I)]. Target bacteria had fewer than three mismatches with each probe. The probe EUB338, specific for the domain Bacteria (Amann *et al.*, 1990), and the negative control probe NON338 (Wallner *et al.*, 1993), were also used.

***In situ* hybridization.** Paraformaldehyde (12%, w/v, in PBS) was added to the collected samples to a final concentration of 3% (w/v) and cells were fixed at 4 °C overnight. Cells were captured on polycarbonate filters (Millipore; pore size 0.2 µm) which were then transferred to centrifuge tubes (Iwaki Glass; volume 50 ml), and resuspended in 2 ml double-distilled water. Bacterial cells from river water samples were similarly concentrated.

HNPP-FISH of river water samples was performed by a modification of the method described in our previous report (Yamaguchi *et al.*, 1996). Twenty microlitres of fixed cell suspension was spotted onto gelatin-coated slides [0.1% gelatin, 0.01% KCr(SO₄)₂], and dried under vacuum. After dehydration in 50, 80, and 100% (v/v) ethanol (3 min each), samples were incubated with lysozyme (0.5 mg ml⁻¹ in 100 mM Tris/HCl pH 8.2, 50 mM EDTA) at 4 °C for 15 min, rinsed with double-distilled water, and dehydrated as described above. Permeabilization was promoted by treatment with proteinase K at a final concentration of 0.1 µg ml⁻¹ in TE buffer (10 mM Tris/HCl pH 8.2, 1 mM EDTA) for 3 min at room temperature and samples were rinsed and dehydrated as described above.

The cells were hybridized in 20 µl hybridization buffer [0.9 M NaCl, 5 mM EDTA, 0.1% SDS, 20 mM Tris/HCl pH 7.5, 30–45% (v/v) formamide] containing 200 ng digoxigenin-labelled probe at 37 °C for 1 h. Hybridization was terminated by sequential washes in double-distilled water at 37 °C, room

Table 1. Physico-chemical characteristics of river water samples from the two sampling sites

Property	Means $\pm$ SD for all 1995–96 measurements	
	Takayama	Kitahashi
Ambient temp. ($^{\circ}$ C)	8.3 ± 7.9	14 ± 9.9
Water temp. ($^{\circ}$ C)	8.0 ± 5.6	16 ± 7.8
pH	7.9 ± 0.4	7.2 ± 0.4
Electrical conductivity (μ S cm^{-1})	120 ± 40	470 ± 250
Total organic carbon (p.p.m.)	2.0 ± 1.3	10 ± 7.9
TDC (cells ml^{-1})*	$3.0 \pm 1.7 \times 10^5$	$9.9 \pm 7.7 \times 10^6$
C.f.u. (cells ml^{-1})†	$1.3 \pm 0.8 \times 10^4$	$2.5 \pm 2.6 \times 10^6$

* Total direct counts determined by DAPI staining.

† Colony-forming units on R2A medium.

Fig. 2. Evolutionary distance tree based on 16S rRNA sequences comparing the five bacterial groups for which probes were designed in this study, and various other bacteria. The tree was constructed from evolutionary distance values with the fastDNAmI program. FC, *Flavobacterium*–*Cytophaga*; BPA, *Burkholderia*–*Pseudomonas* (rRNA III)–authentic *Alcaligenes* group bacteria belong to the beta subclass of *Proteobacteria*; Ac, *Acinetobacter*; VA, *Vibrio*–*Aeromonas*; P(I) *Pseudomonas* (rRNA I) group bacteria belong to the gamma subclass. The horizontal scale bar corresponds to 0.1 nucleotide change per position.

temperature and 4°C . Thirty microlitres of blocking buffer [30 mg ml^{-1} bovine albumin (Wako Pure Chemicals Industries) in PBS] was spotted onto the preparations on ice and then incubated at room temperature for 30 min. Fifteen microlitres of the blocking buffer was replaced by 15 μl alkaline phosphatase-conjugated anti-digoxigenin Fab frag-

ments (Boehringer) diluted 1:30 in blocking buffer (375 milliunits per sample) containing Tween 20 (final concentration 1.0%) and subsequently incubated at room temperature for 45 min. After washing twice in buffer 1 (100 mM Tris/HCl pH 7.5, 150 mM NaCl, 0.05% Tween 20) for 10 min, they were treated in buffer 2 (100 mM Tris/HCl

Table 2. Oligonucleotide probes designed by multiple sequence alignment

Probe name	Specificity	Oligonucleotide sequence	Location
FC	<i>Flavobacterium</i> – <i>Cytophaga</i>	5'-AGGTA CCCCC AGCTT CCATG GCT-3'	1408–1430*
BPA	<i>Burkholderia</i> – <i>Pseudomonas</i> (rRNA III)–authentic <i>Alcaligenes</i>	5'-GTGTG CCGGT TCTCT TTCGA GCAC-3'	1022–1044*
Ac	<i>Acinetobacter</i>	5'-GCGCC ACTAA AGCCT CAAAG GCC-3'	836–858*
VA	<i>Vibrio</i> – <i>Aeromonas</i>	5'-ACGAC GCACT TTTTG GGATT CGCTC ACTAT CGCAA G-3'	1265–1297*
P(I)	<i>Pseudomonas</i> (rRNA I)	5'-ATTTC AGCCT ACCAC CTAA-3'	1467–1486†

* Homologous position on *E. coli* 16S rRNA.† Homologous position on *E. coli* 23S rRNA.

pH 8.0, 100 mM NaCl, 10 mM MgCl₂) for 10 min twice, and incubated in HNPP/Fast Red TR (Boehringer) solution (100 µg HNPP ml⁻¹, 250 µg Fast Red TR ml⁻¹ in buffer 2) for 60 min at room temperature. After hybridization, complete drying was avoided, in order to limit high background signals. The samples were counter-stained with DAPI (4',6-diamidino-2-phenylindole) after washing with double-distilled water, and mounted in MacIlvaine buffer (53.2 mM citric acid, 93.6 mM Na₂HPO₄ pH 4.5) for observation by epifluorescence microscopy (BH-2; Olympus) under blue excitation (excitation filter BP490+EY455; dichroic mirror DM500; absorption filter O515) and UV excitation (excitation filter UG1; dichroic mirror DM400; absorption filter L420). For each slide, at least 3000 cells in different fields of view were counted. The microscopic enumeration results were in all cases obtained from three parallel samples with counts of more than 10000 cells.

Evaluation of probe specificity. The specificity of each designed probe was evaluated by autoradiography with 30 strains. Colony hybridization with ³²P-labelled probe was performed by a modification of the method described by Berger & Kimmel (1987). Hybridization was done at 42 °C for 2 h in a solution containing 100 ng labelled probe DNA with a specific activity of 1 × 10⁸ c.p.m. µg⁻¹, 5 × SSC, 0.1% SDS, tenfold-diluted blocking reagent (Amersham), 0.1% blocking component (Amersham) and 100 µg denatured salmon sperm DNA ml⁻¹. Washing for the EUB and BPA probes was at 65 °C with 1 M NaCl. Washing for the Ac probe was at 65 °C with 0.5 M NaCl. Washing for the P(I), VA and FC probes was at 70 °C with 1, 0.5 and 0.25 NaCl, respectively. The specificity of the designed probes was also tested with whole fixed cells of 30 strains by HNPP-FISH.

RESULTS AND DISCUSSION

Specificity of the designed probes

The target groups of the newly designed probes are shown in Fig. 2. Probe Ac is the only genus-specific probe. *Pseudomonas* (rRNA I) bacteria have no specific region on the 16S rRNA sequence which can be discriminated from close relatives; thus the 23S rRNA sequence was preferred for design of a specific probe. The specificity of the probes was evaluated by colony hybridization with the bacterial strains listed in Table 3.

Optimal washing conditions to maximize specificity for each probe were determined. Under appropriate hybridization and washing conditions, each probe bound only to the rRNAs of the predicted target strains and not to the non-target strains (data not shown).

Sensitive identification of individual bacterial cells by HNPP-FISH has been reported previously (Yamaguchi *et al.*, 1996). In this study, fluorescent signals were enhanced and background lowered by the following improvements: (i) introduction of proteinase K treatment; (ii) addition of Tween 20 to the antigen/antibody reaction buffer; (iii) increase of antibody concentration in the antigen/antibody reaction. The improved method was applied to evaluate the suitability of digoxigenin-labelled probes and to analyse community structure in natural samples.

The specificity of the designed probes was tested by *in situ* hybridization. The stringency of hybridization was adjusted by gradually increasing (in 5% intervals) the formamide concentration in the hybridization buffer while keeping ionic strength (0.9 M NaCl) and hybridization temperature (37 °C) constant. Specificity and hybridization conditions with the five group-specific probes are shown in Table 3. The results show that each probe detected target bacteria even with one, two or three mismatches.

Recovery rate of bacterial cells

The change in absolute numbers of cells during the individual steps of *in situ* hybridization was estimated. When bacterial cells from the polycarbonate filter were resuspended, recovery of cells on to the gelatin-coated slides approached 100% in Kitahashi samples; recovery was 74–90% in Takayama samples. Neither the lysozyme nor the proteinase K step influenced the cell counts of samples under our conditions. The loss of bacterioplankton cells on gelatin-coated slides during all experimental steps was estimated by comparing the cell number obtained by digital images of the same microscopic fields captured by cooled CCD camera before

Table 3. Specificity of the designed oligonucleotide probes determined by *in situ* hybridization

Strain	Presence of signal (no. of mismatches) for probe*:				
	FC [23]	BPA [24]	Ac [23]	VA [36]	P(I) [20]
<i>Chryseobacterium meningosepticum</i> ATCC 13253	+ (1)	– (12)	– (10)	– (19)	– (ND)
<i>Flavobacterium breve</i> (<i>Empedobacter brevis</i>) GIFU3159	+ (ND)	– (ND)	– (ND)	– (ND)	– (ND)
<i>Flavobacterium johnsoniae</i> ATCC 17061	+ (2)	– (7)	– (12)	– (12)	– (ND)
<i>Sphingobacterium thalpophilum</i> ATCC 43320	+ (2)	– (13)	– (11)	– (15)	– (ND)
<i>Alcaligenes faecalis</i> ATCC 19018	– (ND)	+ (ND)	– (ND)	– (ND)	– (9)
<i>Alcaligenes xylosoxidans</i> ATCC 27061	– (ND)	+ (ND)	– (ND)	– (ND)	– (ND)
<i>Burkholderia cepacia</i> ATCC 25416	– (9)	+ (1)	– (8)	– (13)	– (10)
<i>Comamonas acidovorans</i> IAM 12409	– (ND)	+ (ND)	– (ND)	– (ND)	– (ND)
<i>Comamonas testosteroni</i> IAM 12419	– (ND)	+ (ND)	– (ND)	– (ND)	– (ND)
<i>Acinetobacter baumannii</i> ATCC 19606	– (9)	– (10)	+ (0)	– (14)	– (ND)
<i>Acinetobacter calcoaceticus</i> ATCC 23055	– (9)	– (10)	+ (0)	– (10)	– (9)
<i>Acinetobacter haemolyticus</i> ATCC 17906	– (9)	– (10)	+ (0)	– (10)	– (ND)
<i>Acinetobacter johnsonii</i> ATCC 17909	– (9)	– (10)	+ (0)	– (10)	– (ND)
<i>Acinetobacter junii</i> ATCC 17908	– (9)	– (10)	+ (0)	– (14)	– (ND)
<i>Acinetobacter lwoffii</i> ATCC 15309	– (9)	– (10)	+ (0)	– (11)	– (ND)
<i>Aeromonas caviae</i> JCM 1060	– (10)	– (11)	– (12)	+ (2)	– (ND)
<i>Aeromonas hydrophila</i> ATCC 7966	– (9)	– (13)	– (11)	+ (2)	– (10)
<i>Vibrio campbellii</i> ATCC 25920	– (10)	– (9)	– (10)	+ (1)	– (ND)
<i>Vibrio parahaemolyticus</i> ATCC 17802	– (10)	– (8)	– (9)	+ (3)	– (ND)
<i>Vibrio vulnificus</i> ATCC 27562	– (10)	– (8)	– (10)	+ (3)	– (ND)
<i>Pseudomonas mendocina</i> ATCC 25411	– (9)	– (13)	– (7)	– (14)	+ (ND)
<i>Pseudomonas fluorescens</i> RIMD 1615005	– (ND)	– (ND)	– (ND)	– (ND)	+ (ND)
<i>Pseudomonas putida</i> ATCC 12633	– (9)	– (10)	– (6)	– (15)	+ (ND)
<i>Alcaligenes eutrophus</i> (<i>Ralstonia eutropha</i>) IAM 12305	– (ND)	– (ND)	– (ND)	– (ND)	– (ND)
<i>Escherichia coli</i> K-12 W3110	– (12)	– (11)	– (11)	– (8)	– (6)
<i>Moraxella bovis</i> IAM 12313	– (ND)	– (ND)	– (ND)	– (ND)	– (ND)
<i>Moraxella lacunata</i> IAM 12150	– (ND)	– (ND)	– (ND)	– (ND)	– (ND)
<i>Moraxella nonliquefaciens</i> IAM 12314	– (ND)	– (ND)	– (ND)	– (ND)	– (ND)
<i>Pseudomonas</i> (<i>Breviundimonas</i>) <i>diminuta</i> GIFU657	– (ND)	– (ND)	– (ND)	– (ND)	– (ND)
<i>Xanthomonas</i> (<i>Stenotrophomonas</i>) <i>maltophilia</i> KM 1168	– (ND)	– (ND)	– (ND)	– (ND)	– (ND)
Formamide concn (%)	30	40	40	45	45

* Probe lengths are shown in square brackets (see also Table 2).

ND, No data: sequence data are not available in GenBank.

enzymic treatment and after all procedures. More than 90 % of the DAPI-stained cells remained on the slide.

Bacterial community structure

In the samples from Kitahashi (the eutrophic site), the percentages of DAPI-stained cells that could be visualized microscopically with EUB338 were 68 ± 2 %

(29 Jan), 59 ± 5 % (18 Apr) and 54 ± 1 % (1 Oct). In the sample from Takayama (the oligotrophic site) the percentages were 45 ± 3 % (29 Jan), 40 ± 3 % (18 Apr) and 39 ± 1 % (9 Oct) of all DAPI-stained cells hybridized with EUB338 (Fig. 3). The counts with the negative control probe (NON338) accounted for less than 0.5 % of the DAPI counts in both samples. The visualization of resident bacteria hybridizing with EUB338 is shown in

Fig. 3. Comparison of microbial community structure in river water samples collected at Takayama (oligotrophic site) and Kitahashi (eutrophic site) in winter, spring and autumn of 1996. See Table 2 for probe specificities.

Fig. 4. The bright orange staining of bacteria facilitated reliable enumeration (Fig. 4a, c). Counterstaining hybridized cells with DAPI allowed enumeration within the same preparation of both the total number of bacterial cells and the cells that were detected by a given probe (Fig. 4b, d).

Definitive studies correlating environmental conditions with microbial community composition in natural samples are difficult because, until recently, few methods have been available for identifying and quantifying specific bacteria or bacterial groups in the environment without cultivation. In the present study, HNPP-FISH, which we recently used for the sensitive identification of cultured cells (Yamaguchi *et al.*, 1996), was applied successfully to the analysis of complex microbial communities in natural river water with differing levels of pollution. Application of standard FISH with mono-FITC-labelled oligonucleotide probes to river water samples from Kitahashi was less successful; fewer than 20% of all DAPI-stained cells were positive with EUB338 (data not shown). Sensitive *in situ* hybridization with HNPP/Fast Red TR, as employed here, amplifies the fluorescent signal of hybridized bacterial cells by an enzymic reaction. Recently, hybridization with Cy3-labelled oligonucleotide probes in the natural aquatic

environment has been reported (Alfreider *et al.*, 1996; Glöckner *et al.*, 1996) to give detection yields in a similar range to those reported here.

Interestingly, the percentage of total bacteria detectable with EUB338 differed at Kitahashi (the eutrophic site) and Takayama (the oligotrophic site). This is probably due to differences in the abundance of bacteria with enough rRNA for detection. The general metabolic activity of bacterioplankton is presumably higher in a eutrophic environment. For example, Kirchman *et al.* (1985) demonstrated that levels of uptake of radio-isotope-labelled substrates as an index of protein synthesis were higher at sites where the micro-organisms were exposed to higher levels of pollutants, and we found that estimates of physiologically active bacteria in eutrophic rivers were higher than those in oligotrophic rivers (Yamaguchi *et al.*, 1997). At eutrophic sites, bacteria may be growing faster and have a higher rRNA content.

Hybridization with the five group-specific probes resulted in straightforward detection of stained individual cells. Group-specific probe counts for both samples are given as the percentage of DAPI-stained cells in Fig. 3. In the sample from Kitahashi on 29 January, FC ($30 \pm 3\%$) and BPA ($13 \pm 1\%$) counts predominated over Ac ($3 \pm 0.5\%$), VA ($2 \pm 0.5\%$) and P(I) ($2 \pm 1\%$) counts. The community structure did not change appreciably at other sample dates (Fig. 3). Community composition in the sample from Takayama on 25 January was $11 \pm 3\%$ FC, $10 \pm 1\%$ BPA, $8 \pm 2\%$ Ac, $5 \pm 2\%$ VA and $5 \pm 0.5\%$ P(I). Similar results were obtained from the samples of 18 April and 9 October (Fig. 3).

The thorough analysis of microbial community structures requires hybridization with nucleic acid probes for specific populations in the environment of interest. Wagner *et al.* (1994) applied oligonucleotide probes specific for the genus *Acinetobacter* for *in situ* monitoring in activated sludge. Ramsing *et al.* (1993, 1996) used specific probes for sulfate-reducing bacteria to help elucidate the role of the bacteria in the environment. In the present study, five specific probes for certain bacterial groups that are often reported to exist in the natural aquatic environment were designed and employed to analyse microbial community structure in river water. The sum of all bacteria classified by these probes was more than 50% of the DAPI-stained cells in the sample from Kitahashi and 25–38% from Takayama. Most of the EUB338-detectable cells could be identified with the five group-specific probes (Fig. 3). The oligonucleotide probes in this study thus provide a substantial amount of information on bacterial community structure in the river water ecosystem. We believe that this study is the first to closely examine the community composition of the bacteria in river water. Rivers are important ecosystems but the characterization of their indigenous bacterial populations has been comparatively neglected.

Many researchers have investigated the taxonomic distribution and seasonal fluctuation of bacterial strains

Fig. 4. Typical micrographs of bacterial cells detected by HNPP/Fast Red TR whole-cell hybridization: cells hybridized with EUB338 in river water samples collected from Kitahashi (a, b) and Takayama (c, d). Only bacteria detected by the probe showed the orange fluorescence of HNP/TR under blue excitation. The same microscopic fields are shown with UV excitation (b, d) and Blue excitation (a, c). Magnification $\times 850$.

in natural aquatic systems on the basis of culture-dependent methods. A predominance of *Flavobacterium* in river and lake samples has been reported in several studies (Lighthart, 1975; Konda & Tezuka, 1979; Maeda, 1980; Stopinski, 1981; Nuttall, 1982; Sugita *et al.*, 1982; Flint, 1985). The detection of *Flavobacterium* as a predominant genus is consistent with our results: *Flavobacterium*–*Cytophaga* (FC) group bacteria dominated the bacterial community at both sites throughout the sampling period. FC-group bacteria may be common in freshwater systems, and sometimes constitute a

considerable cell fraction. Analysis of the physiology of members of the predominant genera will increase our understanding of their roles in nature, and studies combining modern molecular and traditional cultivation-based techniques should give new insights into the ecology of rivers.

This study demonstrated the feasibility of HNPP-FISH analysis of environmental samples with group-specific oligonucleotide probes. However, 30–40 % of the DAPI-stained cells in the sample from Kitahashi, and approxi-

mately 60% in the sample from Takayama, still could not be identified by HNPP/Fast Red TR whole-cell hybridization with EUB338. These cells may have been impermeable to probes or anti-digoxigenin Fab fragments conjugated to alkaline phosphatase (>244 kDa), or perhaps did not have a ribosomal content high enough for detection by this technique. Cells which are impermeable to probes or antibody, may be resistant to lysozyme or proteinase K treatment. Certain Gram-positive bacteria, e.g. *Staphylococcus* spp., were not sensitive to these enzymes under our conditions. The application of other enzymic or chemical treatments may be required for *in situ* identification of such bacteria, and the detection of larger phylogenetic entities remains limited even with an improved HNPP/Fast Red TR system because cell wall structure and thus probe accessibility may differ between individual species.

Cells with low metabolic activity may be detected by *in situ* PCR. Hodson *et al.* (1995) successfully applied this technique to bacteria and demonstrated that prokaryotic *in situ* PCR enabled single cells within complex mixtures to be not only identified but also characterized genetically. The combination of an HNPP/Fast Red TR detection system with prokaryotic *in situ* PCR could provide information about specific genes and their transcripts at the single-cell level (Kurokawa *et al.*, 1997). Moreover, the sensitivity of the *in situ* PCR technique could be further improved by employing high-sensitivity optical instruments such as the cooled CCD camera (Ramsing *et al.*, 1996).

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