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DNA Microarray Analysis of Temporal and Spatial Variation of Bacterial Communities in Japanese Rivers

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Abstract

To determine the spatial and seasonal variations in bacterial community structure and abundance in the small and steep rivers typically present in Japan, bacterial populations in two rivers, the Yodo River and Kita River, were investigated using a DNA microarray technique. A total of 24 river water samples seasonally collected from four stations in the Yodo River and two stations in the Kita River were analyzed by an oligonucleotide DNA microarray targeting the conserved region of 16S rDNA in 1016 bacterial species. The phyla *Proteobacteria, Firmicutes, Actinobacteria, Cyanobacteria*, and *Bacteroidetes* were the dominant bacterial groups in the river water samples investigated, and alpha-*Proteobacteria* appeared to be the most dominant among the *Proteobacteria*. Overall diversity, composition and shifts in the bacterial communities depended mainly on the season. Pollution level (as indicated by nutrient concentration) and specific bacterial sources, such as effluent from wastewater treatment plants and backflow of seawater, also appeared to influence bacterial community structure in these small, fast-flowing rivers.

Key words: Bacterial community, DNA microarray, river water environment, temporal and spatial variation

INTRODUCTION

Microbial communities play pivotal roles in breaking down organic matter and remineralizing nutrients; these actions strongly influence energy flux and elemental and material circulation in aquatic ecosystems. Microbial populations in the natural environment fluctuate spatiotemporally with changes in the chemical and physical conditions of the surrounding environment[1-2]: some species are persistent or enhanced under a specific condition, whereas others are suppressed or even eliminated under the same condition. In addition, inflow of wastewaters containing xenobiotic and toxic chemicals causes a drastic shift in microbial community structure; this shift may influence the ecological functions of the environment[3-6]. Thus, microbial flora can serve as a bioindicator for estimating water quality[7-8] and environmental soundness.

The influence of environmental conditions on fluctuations in entire microbial communities and on specific microbial populations in riverine environments has been studied over many years. These previous studies have shown that anthropogenic influx of nutrients and xenobiotic pollutants into river water leads to a shift in the structure of the river's entire microbial community[9-10]. Moreover, the overall status of water quality indices influences the specific bacterial populations occurring in natural terrestrial ecosystems[11-12]. However, most studies on the correlation between fluctuation in microbial populations

and environmental variables in riverine environments have focused on large rivers with slow water flow; few studies have examined the types of small and steep rivers that are common in Japan. Because of differences in hydraulic retention time, the influence of environmental conditions on bacterial community structure in these small rivers may be different from that in large rivers. Thus, studies of the spatiotemporal variation of bacterial communities in these small rivers which are typical to the geographical conditions within Japan are needed.

Because the vast majority of environmental bacteria are unculturable[13], the use of culture-independent molecular techniques is helpful for improving our understanding of total microbial communities in the environment. Several techniques such as PCR – denaturing/temperature gradient gel electrophoresis[14-15], terminal restriction fragment length polymorphism[16] and DNA microarray analysis[17] have been developed and applied in the microbial ecology field. Among them, DNA microarray technology is a powerful tool for simultaneously detecting tens or hundreds of thousands of genes in a single sample, thus enabling the detailed analysis of complex microbial communities in the environment. Very recently, the method has been extensively applied to studies of river water microbial ecology[18-21].

Here, river water samples were collected seasonally from stations on two rivers with different pollution levels in the Kinki district of Japan; we used a DNA microarray technique to analyze the bacterial communities in the samples. The DNA microarray consisted of oligonucleotide probes targeting the conserved region of 16S rDNA in 1016 bacterial species found most commonly in the environment. From these results, we characterized the temporal and spatial variations in the riverine bacterial communities. Our monitoring rivers, Yodo River and Kita River, are small and steep rivers with a short hydraulic retention time as compared with continental rivers abroad. Furthermore, even Yodo River, the larger river of the two monitoring rivers, ranks the 79th in the river extension among first-class rivers in Japan. Thus, results of this study can represent the general variation of bacterial community in small rivers typically present in Japan.

MATERIALS AND METHODS

Study sites and sampling locations   Surface water samples were collected from two stations on the Kita River (K1 and K2; upstream and downstream, respectively) and four stations on the Yodo River (Y1, Y2, Y3, and Y4; upstream to downstream) in the Kinki district of Japan (Fig. 1) in October 2005 (autumn), August 2006 (summer), January 2007 (winter), and May 2007 (spring). The Kita River has clean water and is in a rural setting with relatively low pollution levels, whereas the Yodo River is
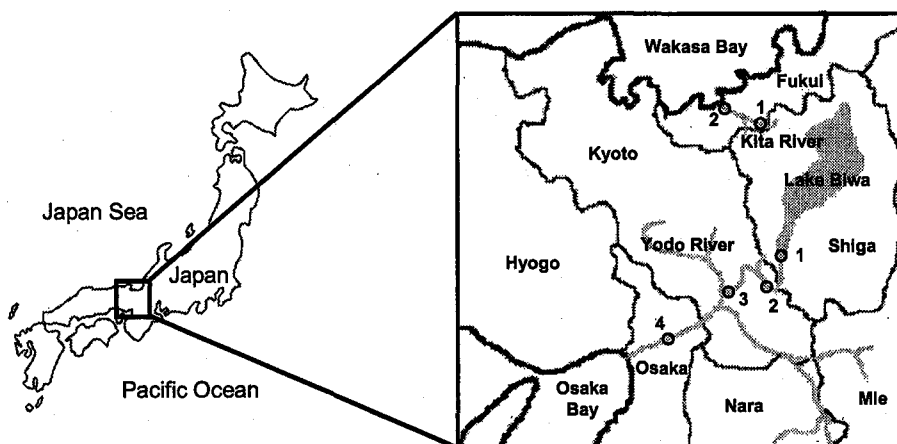

Fig. 1 Locations of sampling stations

the largest river in the Kinki district and receives large amounts of effluent from municipal wastewater treatment plants (WWTPs) and other industries in the surrounding urban areas. River water samples collected were transported on ice to the laboratory and subjected to water quality analysis on the same day and DNA extraction within 12 h. Water temperature, electrical conductivity, pH, and dissolved oxygen (DO) were recorded at the sampling site. Concentrations of dissolved organic carbon (DOC), total nitrogen (T-N), total phosphorus (T-P), culturable heterotrophic bacteria (CHB), and eubacterial 16S rDNA were analyzed in the laboratory. DOC was analyzed with a total organic carbon analyzer (TOC-5000A; Shimadzu, Kyoto, Japan). Concentrations of T-N and T-P were measured by the alkaline potassium peroxodisulfate decomposition – ultraviolet absorptiometry method and the potassium peroxodisulfate decomposition – molybdenum blue (ascorbic acid) absorptiometry method, respectively[22]. Numbers of CHB were determined by plating on a 1/10 dilution of CGY medium[23]. Eubacterial 16S rDNA number was enumerated by most-probable-number PCR[24] using an EUBf933 and EUBr1387 primer set[25].

DNA microarray analysis   DNA was extracted from each river water sample by a combination of cell lysis with proteinase K and phenol–chloroform extraction, as described previously[26]. The conserved region of eubacterial 16S rDNA (approximately 510 bp) was PCR amplified using an 8UA (5′-AGA GTT TGA TCM TGG CTC AG-3′) and 519B (5′-GTA TTA CCG CGG CKG CTG-3′) primer set. The 5′-end of the reverse primer was labeled with Cy3; thus PCR products were fluorescently labeled at the same time. PCR amplification was performed in a 20-μl PCR mixture containing 1 × Ex *Taq* buffer (TaKaRa, Shiga, Japan), 200 μM dNTPs (TaKaRa), 20 pmol of forward and reverse primers, 0.6 U of *Taq* DNA polymerase (TaKaRa), and 4 μl of DNA template. The thermal profile for PCR amplification included an initial denaturation at 95 ℃ for 3 min; 35 cycles of denaturation at 95 ℃ for 30 s; annealing at 55 ℃ for 30 s, and extension at

72 ℃ for 30 s; and a final extension step at 72 ℃ for 7 min. All PCR amplifications were carried out with a Mastercycler Standard (Eppendorf, Tokyo, Japan). Amplified products were purified by ethanol precipitation.

Microarrays for detecting eubacteria were purchased from AMR Inc. (Gifu, Japan). Oligonucleotide probes for the 16S rRNA gene of 1016 eubacterial species commonly present in the environment were spotted on the microarray slides. The spotted probe number at the phylum level was as follows: *Actinobacteria*, 152; *Bacteroidetes*, 48; *Cyanobacteria*, 39; *Firmicutes*, 226; *Proteobacteria*, 455 (*alpha* subclass, 123; *beta* subclass, 78; *gamma* subclass, 133; *delta* subclass, 108; *epsilon* subclass, 13); Others, 96 (*Acidobacteria*, 3; *Aquificae*, 5; *Chlamydia*, 8; *Chlorobi*, 4; *Chloroflexi*, 9; *Chrysiogenetes*, 1; *Deferribacteres*, 5; *Deinococcus-Thermus*, 3; *Dictyoglomi*, 1; *Fibrobacteres*, 1; *Fusobacteria*, 15; *Nitrospira*, 10; *Placentomycetes*, 4; *Spirochaetes*, 17; *Spiralbacteria*, 1; *Thermodesulfobacteria*, 1; *Thermomicrobia*, 1; *Thermotogae*, 5; *Verrucomicrobia*, 2). The balance of probe numbers in each phylum, e.g. higher probe numbers for *Proteobacteria*, *Firmicutes* and *Actinobacteria* seems to resemble the general bacterial population in aquatic samples reported previously[3, 9, 20–21, 27–28]. Thus, the microarray used here would be reasonable for investigating the riverine microbial community.

Microarray hybridization was performed in accordance with the manufacturer's instructions, as follows. The microarrays were prehybridized in prehybridization buffer (2 × SSC, 0.2% sodium dodecyl sulfate (SDS)) for 15 min at room temperature and in freshly made prehybridization buffer for 5 min at 37 ℃. After the slides had been dipped in ultrapure water three times to remove excess prehybridization buffer, the arrays were dried by centrifugation (110 × *g*, 2 to 4 min) and hybridized immediately with the labeled target DNA. Cy3-labeled target DNA (35 μg) was dissolved in a 50-μl hybridization buffer (5 × SSC, 0.5% SDS), denatured at 90 ℃ for 1 min, cooled down to 55 ℃, and deposited onto a glass coverslip. The prehybridized array was placed on the coverslip and then into a hybridization

chamber (DNA Chip Research Inc., Kanagawa, Japan), where 150 µl of 5 M NaCl was applied to avoid drying. Hybridizations were carried out at 55 ℃ for 16 h. Following hybridization, coverslips were removed by immersion in 2 × SSC, 0.2% SDS at 37 ℃. Arrays were washed with 2 × SSC, 0.2% SDS and with 2 × SSC at room temperature for 1 min each, before being air dried in the dark.

An arrayWoRx (GE Healthcare UK Ltd., Buckinghamshire, England) was used to scan microarray slides in accordance with the manufacturer's instructions. Scanned images were then processed with Array Vision ver. 8.0 (GE Healthcare UK Ltd.). After subtraction of the background intensity, the signal intensities of the spots were normalized by the intensity of the positive Cy3 spots. Test spots whose relative signal intensities (RSI) exceeded 0.1 were considered positive

and used for further analysis.

Statistical analysis The Shannon-Weaver diversity index[29] was calculated using natural logarithm by the following equations:

$$H' = -\sum Pi(\ln Pi),$$

where $Pi = n_i / N$, n_i is the RSI of bacterial species i, and N is the summation of normalized intensities in a sample.

Principal components analysis (PCA) and multivariate analysis were performed with the statistical analysis tool SPSS ver. 15.0 for Windows (SPSS Inc., Chicago, IL, USA).

RESULTS

Quality of river water samples We examined the physicochemical and biological qualities of the river water samples (Table 1). Water temperature varied from 21.6 ℃ to 30.2 ℃ in summer, 11.8 ℃ to 22 ℃ in spring

Table 1 Physicochemical and biological water quality parameters in river water samples[a]

Sampling date	Sampling station	Temp (℃)	pH	DO (mg/l)	Conductivity (mS/cm)	DOC (mg/l)	T-N (mg/l)	Heterotrophic bacteria (CFU/ml)	Eubacterial 16S rDNA (MPN-copies/ml)
October 2005 (autumn)	Y1	21.2	8.1	7.9	0.1	1.5	1.1	2.7×10^4	2.4×10^5
	Y2	21.5	7.2	6.8	0.1	1.1	1.4	7.1×10^3	1.5×10^5
	Y3	22.0	7.3	7.4	0.2	1.9	2.0	1.3×10^4	2.1×10^5
	Y4	21.8	6.6	6.4	0.2	2.0	1.6	3.3×10^4	2.4×10^4
	K1	13.4	6.7	9.5	0.1	0.36	2.4	5.7×10^3	2.3×10^3
	K2	11.8	6.5	6.5	13.9	1.8	2.6	1.9×10^5	7.0×10^3
August 2006 (summer)	Y1	30.0	9.0	6.9	1.4	NA	NA	1.1×10^4	2.3×10^4
	Y2	28.6	7.3	4.8	0.1	NA	NA	1.4×10^4	2.3×10^4
	Y3	29.8	7.5	6.1	0.2	NA	NA	4.0×10^4	9.3×10^4
	Y4	30.2	8.5	6.3	0.1	NA	NA	4.0×10^4	9.3×10^4
	K1	21.6	8.2	9.3	ND	NA	NA	6.3×10^3	2.4×10^4
	K2	30.0	7.8	3.3	50.1	NA	NA	1.1×10^5	1.5×10^4
January 2007 (winter)	Y1	7.2	7.5	7.8	0.1	6.3	0.87	4.6×10^4	9.3×10^3
	Y2	6.5	7.6	8.1	0.1	5.5	0.80	7.8×10^3	4.3×10^3
	Y3	13.1	7.1	5.9	0.3	11.3	4.6	1.7×10^4	2.4×10^4
	Y4	8.4	7.5	4.2	0.1	6.1	1.7	4.4×10^4	9.3×10^3
	K1	6.6	7.2	6.3	ND	2.8	0.67	4.1×10^3	1.5×10^3
	K2	7.1	6.9	6.8	0.1	4.8	0.50	4.4×10^4	2.1×10^3
May 2007 (spring)	Y1	19.8	7.7	9.1	0.1	2.4	0.34	4.6×10^4	2.4×10^6
	Y2	19.4	7.4	7.6	0.1	2.7	0.69	7.9×10^3	2.4×10^4
	Y3	21.0	7.1	7.0	0.2	8.2	1.9	4.7×10^3	9.3×10^6
	Y4	20.5	7.4	7.9	0.1	4.5	1.5	1.8×10^4	9.3×10^4
	K1	12.8	7.2	8.8	0.1	8.2	0.88	4.5×10^3	2.4×10^4
	K2	16.8	6.6	6.2	7.0	3.3	0.90	5.7×10^4	9.3×10^4

[a] NA, not analyzed; ND, not detected.

and autumn, and 6.5 ℃ to 13.1 ℃ in winter. The pH of most samples was around neutral, although several samples had alkaline pH values higher than 8. Electrical conductivity was low (0 to 0.3 mS/cm) in almost all the samples, with the exception of samples from downstream in the Kita River (station K2), which had values of 50.1, 13.9 and 7 (mS/cm) for summer, autumn and spring respectively, indicating that a brackish water environment had developed owing to the backflow of marine water. DO varied widely among samples, ranging from 3.3 to 9.5 mg/l. Concentrations of DOC, CHB, and eubacterial 16S rDNA were lowest upstream in the Kita River (station K1) in most seasons, where the inflow of anthropogenic effluent was presumed lowest among all sampling stations. DOC and CHB increased by 1.7 to 5 times and more than 10 times during the flow from station K1 to station K2 in the Kita River, respectively, possibly because of the inflow of effluent from the surrounding area and the backflow of marine water containing contaminants accumulated in Wakasa Bay. In the Yodo River, concentrations of CHB, DOC, and T-N usually increased between sampling stations Y2 and Y3, indicating that effluent from WWTPs located between these sampling stations largely influenced the water quality at station Y3.

Diversity of entire bacterial community
The numbers of bacterial species detected in DNA microarray analysis varied among samples, ranging from 15 to 807 (Fig. 2). Of the 1016 bacterial species targeted, a total of 854 (84%) were detected in one or more samples.

Higher numbers of bacterial species (610 to 807 species) were detected in summer and autumn, with the exception of an autumn sample from station K1 that had only 159 detectable bacterial species. Winter samples had the lowest numbers of bacterial species, ranging from 15 to 50, although one sample from station K1 contained as many as 524 different species. In spring, the numbers of bacterial species detected at stations K1, K2, Y1, Y2, and Y4 ranged from 362 to 620 — higher than in winter and lower than in autumn and summer — whereas a spring sample from station Y3 contained an
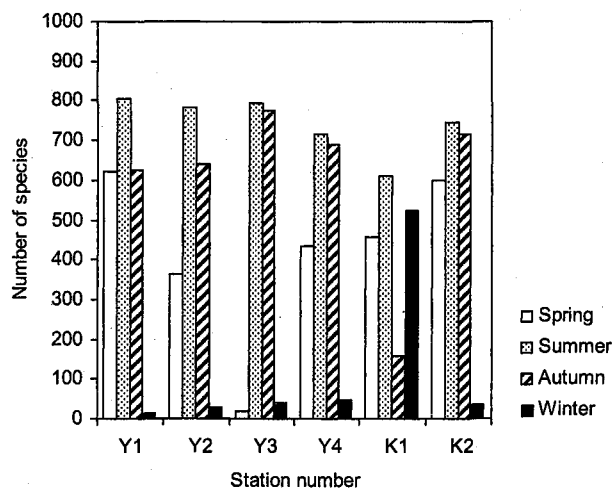

Fig. 2 Numbers of bacterial species detected in water samples from the Kita and Yodo Rivers in four different seasons

exceptionally low number (17 species).

In the Kita River, fewer bacterial species were found at upstream station K1 than at downstream station K2 in spring, summer, and autumn (Fig. 2 and Table 2), and a very sharp increase in the numbers of almost all phyla was observed between K1 and K2 in autumn. In contrast, in winter the community at station K1 had over 10 times more species than at station K2, as indicated above. In the Yodo River, although the number of the bacterial species detected did not drastically change along the flow in summer, autumn, and winter, it increased slightly between stations Y2 and Y3 (Fig. 2 and Table 2). Exceptionally, in spring the number of bacterial species largely decreased from station Y1 (620 species) to station Y3 (17 species) but recovered in Y4 (433 species).

H' calculated from the RSIs of the detected bacterial species (Fig. 3) showed spatiotemporal variation similar to that of the detected number of bacterial species described above.

Bacterial species composition　　Bacterial species detected in the analysis are summarized at the phylum level in Table 2. A total of 854 bacterial species detected were classified into six groups according to their seasonal occurrence patterns (Table 3): Group A consisted of 505 species commonly present in spring, summer, and autumn; group B consisted of 220 species specific to summer

Table 2 Detailed distribution of bacterial species in each sample

Phylum (Total probes)[a]	Spring Y1	Y2	Y3	Y4	K1	K2	Summer Y1	Y2	Y3	Y4	K1	K2	Autumn Y1	Y2	Y3	Y4	K1	K2	Winter Y1	Y2	Y3	Y4	K1	K2
Actinobacteria (152)	77 [51][b]	63 [41]	6 [4]	55 [36]	58 [38]	77 [51]	131 [86]	130 [86]	137 [90]	125 [82]	101 [66]	129 [85]	113 [74]	118 [78]	126 [83]	117 [77]	85 [56]	119 [78]	1 [1]	9 [6]	6 [4]	12 [8]	71 [47]	1 [1]
Bacteroidetes (48)	36 [75]	21 [44]	1 [2]	22 [46]	28 [58]	34 [71]	45 [94]	38 [79]	46 [96]	44 [92]	36 [75]	43 [90]	37 [77]	28 [58]	43 [90]	40 [83]	5 [10]	40 [83]	1 [2]	2 [4]	5 [10]	3 [6]	27 [56]	2 [4]
Cyanobacteria (39)	35 [90]	23 [59]	1 [3]	21 [54]	25 [64]	34 [87]	33 [85]	37 [95]	31 [79]	31 [79]	36 [92]	34 [87]	29 [74]	26 [67]	36 [92]	29 [74]	1 [3]	29 [74]	0 [0]	0 [0]	0 [0]	0 [0]	33 [85]	1 [3]
Firmicutes (226)	174 [77]	86 [38]	1 [0]	113 [50]	114 [50]	165 [73]	180 [80]	167 [74]	166 [73]	141 [62]	114 [50]	143 [63]	126 [56]	134 [59]	173 [77]	149 [66]	14 [6]	145 [64]	6 [3]	7 [3]	8 [4]	9 [4]	144 [64]	11 [5]
Proteobacteria (455)	264 [58]	155 [34]	6 [1]	203 [45]	212 [47]	261 [57]	338 [74]	336 [74]	336 [74]	306 [67]	271 [60]	334 [73]	290 [64]	307 [67]	342 [75]	327 [72]	50 [11]	341 [75]	6 [1]	11 [2]	15 [3]	20 [4]	232 [51]	24 [5]
- Alpha (123)	105 [85]	65 [53]	1 [1]	89 [72]	81 [66]	103 [84]	119 [97]	119 [97]	118 [96]	108 [88]	101 [82]	117 [95]	102 [83]	110 [89]	119 [97]	114 [93]	11 [9]	117 [95]	1 [1]	4 [3]	5 [4]	6 [5]	98 [80]	12 [10]
- Beta (78)	37 [47]	23 [29]	0 [0]	31 [40]	30 [38]	36 [46]	59 [76]	53 [68]	62 [79]	53 [68]	55 [71]	56 [72]	51 [65]	54 [69]	58 [74]	58 [74]	15 [19]	56 [72]	4 [5]	1 [1]	3 [4]	2 [3]	32 [41]	7 [9]
-Gamma (133)	80 [60]	46 [35]	1 [1]	60 [45]	67 [50]	77 [58]	101 [76]	112 [84]	99 [74]	93 [70]	77 [58]	107 [80]	85 [64]	94 [71]	103 [77]	102 [77]	8 [6]	109 [82]	1 [1]	5 [4]	7 [5]	10 [8]	64 [78]	5 [4]
- Delta (108)	37 [34]	19 [18]	3 [3]	21 [19]	31 [29]	39 [36]	47 [44]	44 [41]	45 [42]	45 [42]	33 [31]	42 [39]	44 [41]	45 [42]	50 [46]	45 [42]	15 [14]	47 [44]	0 [0]	1 [1]	0 [0]	2 [2]	33 [31]	0 [0]
- Epsilon (13)	5 [38]	2 [15]	1 [8]	2 [15]	3 [23]	6 [46]	12 [92]	8 [62]	12 [92]	7 [54]	5 [38]	12 [92]	8 [62]	4 [31]	12 [92]	8 [62]	1 [8]	12 [92]	0 [0]	0 [0]	0 [0]	0 [0]	5 [38]	0 [0]
Others (96)	34 [35]	14 [14]	2 [2]	19 [19]	19 [19]	30 [31]	80 [82]	74 [76]	78 [80]	67 [68]	52 [53]	63 [64]	29 [30]	27 [28]	55 [56]	29 [30]	4 [4]	41 [42]	1 [1]	2 [2]	6 [6]	6 [6]	17 [17]	0 [0]
Total (1,016)	620 [61]	362 [36]	17 [2]	433 [43]	456 [45]	601 [59]	807 [79]	782 [77]	794 [78]	714 [70]	610 [60]	746 [73]	624 [61]	640 [63]	775 [76]	691 [68]	159 [16]	715 [70]	15 [1]	31 [3]	40 [4]	50 [5]	524 [51]	39 [4]

[a]Numbers in round parentheses indicate total targeted species.
[b]Numbers in box parentheses indicate percentage of detected probes. Note the numbers are rounded-off to eliminate decimal value.

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and autumn; groups C, D, and E consisted of 44, 6, and 60 species specific to summer, autumn, and spring, respectively; and group F consisted of 19 species randomly present in
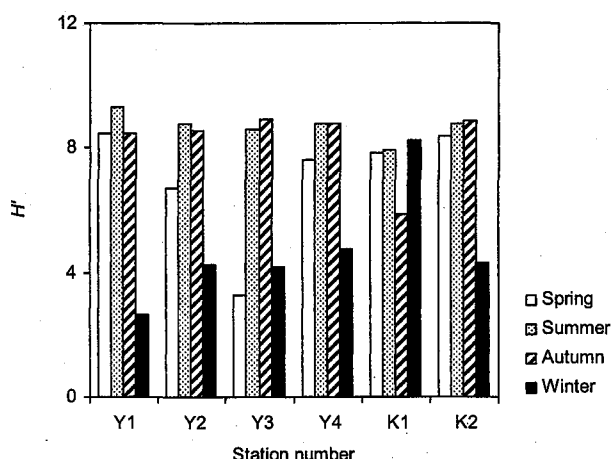

Fig. 3 Seasonal variation in the bacterial diversity of Kita and Yodo Rivers. The Shannon-Weaver index, H', was calculated for each of 24 river water samples by the method described in *Materials and methods*.

one or more seasons.

Five phyla, *Cyanobacteria, Bacteroidetes, Actinobacteria, Proteobacteria* and *Firmicutes* were dominant in the Yodo and Kita Rivers in spring, summer, and autumn with 70%, 68%, 65%, 57% and 57% of the average detection ratio to the total targeted species, respectively, although the most dominant phylum varied depending on the sample (Table 3). In the *Proteobacteria*, the *alpha* subclass (average detection ratio in spring, summer and autumn: 77%) was the most frequently detected followed by the *gamma* (average detection ratio: 59%) and *beta* (average detection ratio: 56%) subclasses. The numbers of species detected among the dominant phyla clearly decreased in the following order: summer > autumn > spring > winter; this was in accordance with the seasonal variation in the total number of bacterial species detected (Fig. 2). Thus, fluctuations in the abundance of the dominant bacterial phyla strongly influenced those of the entire bacterial community.

Principal components analysis   In PCA

Table 3 Classification of detected bacterial species at the phylum level according to seasonal occurrence pattern

Phylum[a]	Pattern						Undetected in any of four seasons
	A	B	C	D	E	F	
	Ubiquitous in spring, summer, and autumn	Specific to summer and autumn	Specific to summer	Specific to autumn	Specific to spring	Random presence in winter and one or more seasons	
Actinobacteria (152)	70 [46][b]	51 [34]	5 [3]	0 [0]	3 [2]	2 [1]	21 [14]
Bacteroidetes (48)	28 [58]	15 [31]	1 [2]	0 [0]	2 [4]	1 [2]	1 [2]
Cyanobacteria (39)	33 [85]	4 [10]	1 [3]	0 [0]	1 [3]	0 [0]	0 [0]
Firmicutes (226)	129 [57]	31 [14]	3 [1]	1 [0]	31 [14]	5 [2]	26 [12]
Proteobacteria (455)	230[51]	93 [20]	10 [2]	5 [1]	14 [3]	10 [2]	93 [20]
– *Alpha* (123)	98 [80]	17 [14]	0 [0]	0 [0]	2 [2]	4 [3]	2 [2]
– *Beta* (78)	31 [40]	20 [26]	2 [3]	2 [3]	1 [1]	4 [5]	18 [23]
– *Gamma* (133)	63 [47]	36 [27]	6 [5]	0 [0]	8 [6]	1 [1]	19 [14]
– *Delta* (108)	33 [31]	13 [12]	2 [2]	3 [3]	3 [3]	1 [1]	53 [49]
– *Epsilon* (13)	5 [38]	7 [54]	0 [0]	0 [0]	0 [0]	0 [0]	1 [8]
Others (96)	15 [15]	26 [27]	24 [24]	0 [0]	9 [9]	1 [1]	23 [23]
Total (1,016)	505 [50]	220 [22]	44 [4]	6 [0]	60 [6]	19 [2]	164 [16]

a Numbers in round parentheses indicate total targeted species.
b Numbers in box parentheses indicate percentage of detected probes. Note the numbers are rounded-off to eliminate decimal value.

based on the data of microarray analysis, almost half (49.6%) of the total variation was explained by extracting the first (PC1) and second (PC2) principal components. Scatter plot based on the PC1 and PC2 revealed that the 24 samples analyzed could be divided into three core groups, A, B, and C (Fig. 4). Core group A consisted of summer and autumn samples; core group B consisted of winter samples (as well as an anomalous sample from K1 and a spring sample from Y3), and core group C consisted of spring samples (as well as an anomalous sample from Y3 and a winter sample from K1). Further PCA performed on the samples classified into core group A clearly separated summer samples from autumn samples (data not shown). These results suggest that the bacterial species composition of the river water changed seasonally. The classification of a winter sample from K1 and a spring sample from Y3 into core groups C and B, respectively, resulted from the tendency for the PCA score which depended on the total number of bacterial species detected in a sample. Similarly, a spring sample from Y3 that had a lower number of bacterial species than the other spring samples was located in core group B, which usually had lower numbers of species; conversely, a winter

sample from K1 that had a higher number of bacterial species than the other winter samples was grouped into core group C. The PC1 values obtained in the PCA (Fig. 4) showed a strong positive correlation with the number of bacterial species detected (Fig. 5). In contrast, no clear relationship was observed between the PC2 values and any of the physicochemical parameters measured.

DISCUSSION

Our microarray analysis targeting approximately 1000 bacterial species in river water samples revealed that the bacterial community in the river water environment varied primarily with the season. Previous studies have also reported a strong influence of seasonal variation in environmental conditions on the bacterial composition of river water environments[10-11]. We detected fewer bacterial species in winter than in the other seasons, probably because most of the dominant bacteria in river water are mesophilic and the cold winter temperatures are unsuitable for their growth. In particular, although the numbers of culturable bacteria were similar in spring and winter (Table 1), the species diversity observed in the microarray analysis was much lower in winter than in spring (Table 2). The
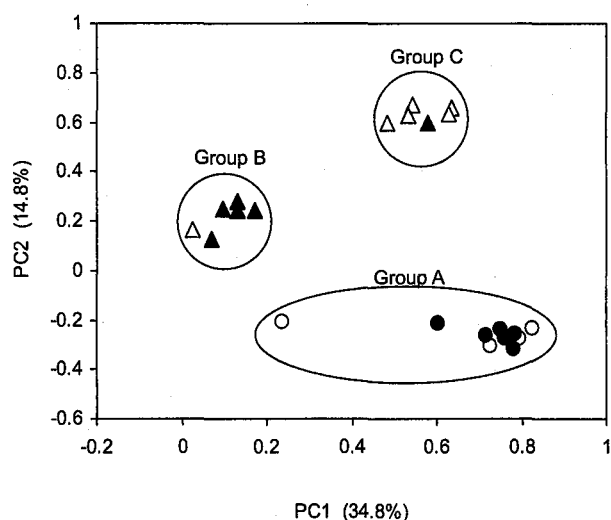

Fig. 4 Principal components (PC) analysis of micro-array analysis data on the Yodo and Kita Rivers. Symbols: open triangles, spring; closed triangles, winter; open circles, autumn; closed circles, summer.
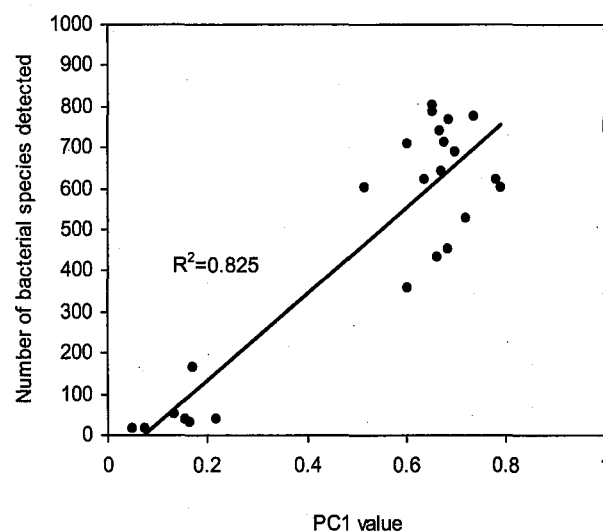

Fig. 5 Correlation between the value of principal component analysis factor 1 (PC1) and the number of bacteria detected in river water samples

differences in 16S rDNA copy number between winter and the other seasons (Table 1) suggest that most of the unculturable bacteria, which may account for >99% of all bacteria in fresh water[13] do not persist under cold conditions. The recording of an exceptionally large number of bacterial species at K1 in winter suggests the presence of largely diverse bacteria at low concentrations and without highly dominant species. At Y3 in spring, despite the large 16S rDNA copy number, the number of detected species was quite low, and their RSI was only marginal. This implies that bacterial species, which are not detected by the microarray used here, were abundantly present in the sample.

In each sampling season, the bacterial species composition at K1, the upstream station on the Kita River, was highly different from that at the downstream station (K2) on the same river and at any stations on the Yodo River. Our measurements of physico-chemical water quality parameters suggested that the upstream part of the Kita River generally had lower pollution levels, although the spring sample had relatively high organic and nitrogen concentrations. A number of previous studies have reported that the input of anthropogenic wastewaters containing various pollutants, including easily degradable organics, nutrients, and xenobiotic compounds, can have affect the composition of riverine microbial communities[4-6, 10, 30]. Thus, the difference in community composition between station K1 and the other stations may be due to a dissimilarity in pollution levels. The slight increase in the number of bacterial species between Y2 and Y3 in the Yodo River (except spring) may have occurred partly because of the growth of some species that were present at undetectable levels upstream and preferred the polluted conditions formed by the discharge of effluent containing various pollutants from the WWTPs located between Y2 and Y3. The elucidation on positive correlation between concentration of some bacterial species and pollution level was previously reported[4]. The inflow of bacterial species in the WWTP effluent itself may also have contributed to the increase in the bacterial diversity between Y2 and Y3[31-32].

Samples from station K2 showed exceptionally higher electrical conductivity in spring, summer, and autumn (7.0, 50.1 and 13.9 mS/cm, respectively) than the other samples (0.1 to 1.4 mS/cm; Table 1). Correspondingly, the bacterial communities in these K2 samples exhibited high species diversity. In contrast, the bacterial diversity at the same station in winter, when the electrical conductivity was surprisingly low (0.1 mS/cm), was the lowest among the samples from the Kita River. In the brackish environment near the mouth of a river, freshwater from the river and backflow from the sea intermix, creating unique conditions with characteristics intermediate between those of freshwater and seawater and with an increased hydraulic retention time. Consequently, a highly divergent microbial community can be established in the brackish environment[9]. Therefore, the high bacterial diversity observed at station K2 in spring, summer, and autumn can be attributed to the formation of such brackish conditions.

Previous studies have reported that bacterial community composition gradually changes in large rivers along the course of flow[21, 28]. Winter et al.[21] suggested that such gradual shifts result from the adaptation of a riverine community to changing environmental conditions over the course of the river. In contrast, in the two rivers investigated here, the bacterial community appeared to respond sharply to specific geographic features and facilities which affect the river water quality rather successively adapt to changing conditions in the course of the water flow. Such differential spatial variation was likely to have been caused by the short retention times of the rivers we monitored.

The phyla *Proteobacteria*, *Firmicutes*, *Actinobacteria*, *Cyanobacteria*, and *Bacteroidetes* were found to be the dominant bacterial groups in the two rivers. *Proteobacteria*, *Firmicutes*, and *Cytophaga–Flavobacterium–Bacteroidetes* have been commonly detected as dominant bacterial groups in riverine environments[3-4, 9, 28]. In the phylum *Proteobacteria*, the *beta* subclass has been observed as dominant in freshwater and the *alpha* subclass as dominant in seawater[9]. In contrast, we found that the *alpha Proteobacteria* were dominant in the two rivers

analyzed. Although the reason for the discrepancy has not been completely elucidated, this may be a local characteristic of the rivers we monitored.

In conclusion, our study revealed spatiotemporal variation in the bacterial communities occurring in two small and steep rivers typical of those present in Japan. Seasonal variables most strongly affected the bacterial community, although geographical characteristics, including pollution level and specific sources (effluent from WWTPs and backflow of seawater), were also significant influences. We targeted free-living bacteria in order to investigate the spatiotemporal changes in bacterial communities in the river environment. However, it has been pointed out that particle-attached biofilm bacteria are also an important part of the microbial ecosystem in riverine environments[7, 9, 33-34]. Thus, further studies focused on biofilm bacteria, including clarification of the relationship between biofilm bacteria and the free-living bacteria analyzed here, are needed if we are to thoroughly understand the spatiotemporal variations in riverine bacterial communities.

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REFERENCES

1) Branco, R., Chunga, A.P., Verissimob, A., and Morais, P.V.: Impact of chromium-contaminated wastewaters on the microbial community of a river. FEMS Microbiol. Ecol., 54, 35-46 (2005)

2) Chénier, M.R., Beaumier, D., Roy, R., Driscoll, B.T., Lawrence, J.R., and Greer, C.W.: Impact of seasonal variations and nutrient inputs on nitrogen cycling and degradation of hexadecane by replicated river biofilms. Appl. Environ. Microbiol., 69, 5170-5177 (2003)

3) Brummer, I.H.M., Fehr, W., and Wagner-Dobler, I.: Biofilm community structure in polluted rivers: abundance of dominant phylogenetic groups over a complete annual cycle. Appl. Environ. Microbiol., 66, 3078-3082 (2000)

4) Feris, K.P., Ramsey, P.W., Frazar, C., Rillig, M., Moore, J.N., Gannon, J.E., and Holben, W.E.: Seasonal dynamics of shallow-hyporheic-zone microbial community structure along a heavy-metal contamination gradient. Appl. Environ. Microbiol., 70, 2323-2331 (2004)

5) Fossi, M.C., Focardi, S., Leonzio, C., Gavilan, J.F., Barra, R., and Parra, O.: Use of biomarkers to evaluate effects of xenobiotic compounds in the Biobio basin (Central Chile). Bull. Environ. Contam. Toxicol., 55, 36-42 (1995)

6) Rodriguez, V., Aguirre de Carcer, D., Loza, V., Perona, E., and Mateo, P.: A molecular fingerprint technique to detect pollution-related changes in river cyanobacterial diversity. J. Environ. Qual., 36, 464-468 (2007)

7) Brummer, I.H.M., Felske, A., and Wagner-Dobler, I.: Diversity and seasonal variability of beta-proteobacteria in biofilms of polluted rivers: analysis by temperature gradient gel electrophoresis and cloning. Appl. Environ. Microbiol., 69, 4463-4473 (2003)

8) Douterelo, I., Perona, E., and Mateo, P.: Use of cyanobacteria to assess water quality in running waters. Environ. Pollut., 127, 377-384 (2004)

9) Crump, B.C., Armbrust, E.V., and Baross, J.A.: Phylogenetic analysis of particle-attached and free-living bacterial communities in the Columbia River, its estuary, and the adjacent coastal ocean. Appl. Environ. Microbiol., 65, 3192-3204 (1999)

10) Rubin, M.A., and Leff, L.G.: Nutrients and other abiotic factors affecting bacterial communities in an Ohio river (USA). Microb. Ecol., 54, 374-383 (2007)

11) Bell, C.R., Holder-Franklin, M.A., and Franklin, M.: Correlations between predominant heterotrophic bacteria and physicochemical water quality parameters in two Canadian rivers. Appl. Environ.

Microbiol., 43, 269-283 (1982)

12) Hirayama, H., Takai, K., Inagaki, F., Yamamoto, Y., Suzuki, M., Nealson, K.H., and Horikoshi, H.: Bacterial community shift along a subsurface geothermal water stream in a Japanese gold mine. Extremophiles, 9, 169-184 (2005)

13) Amann, R.I., Ludwig, W., and Schleiter, K.H.: Phylogenetic identification and in situ detection of individual microbial cells without cultivation. Microbiol. Rev., 59, 143-169 (1995)

14) Muyzer, G., de Waal, E.C., and Uitterlinden, A.G.: Profiling of complex microbial population by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rDNA. Appl. Environ. Microbiol., 59, 695-700 (1993)

15) Riesner, D., Steger, G., Zimmat, R., Owens, R.A., Wagenhöfer, M., Hillen, W., Vollbach, S., and Henco, K.: Temperature-gradient gel electrophoresis of nucleic acids: analysis of conformational transitions, sequence variations, and protein-nucleic acid interactions. Electrophoresis, 10, 377-389 (1989)

16) Liu, W.T., Marsh, T.L., Cheng, H., and Forney, L.J.: Characterization of microbial diversity by determining terminal restriction fragment length polymorphisms of genes encoding 16S rRNA. Appl. Environ. Microbiol., 63, 4516-4522 (1997)

17) Guschin, D.Y., Mobarry, B.K., Proudnikov, D., Stahl, D.A., Rittmann, B.E., and Mirzabekov, A.D.: Oligonucleotide microchips as genosensors for determinative and environmental studies in microbiology. Appl. Environ. Microbiol., 63, 2397-2402 (1997)

18) DeSantis, T.Z., Stone, C.E., Murray, S.R., Moberg, J.P., and Anderson, G.L.: Rapid quantification and taxonomic classification of environmental DNA from both prokaryotic and eukaryotic origins using a microarray. FEMS Microbiol. Lett., 245, 271-278 (2005)

19) Loy, A., Schulz, C., Lucker, S., Schopfer-Wendels, A., Stoecker, K., Baranyi, C., Lehner, A., and Wagner, M.: 16S rRNA gene-based oligonucleotide microarray for environmental monitoring of the betaproteobacterial order "Rhodocyclales". Appl.

Environ. Microbiol., 71, 1373-1386 (2005)

20) Peplies, J., Lachmund, C., Glockner, F.O., and Manz, W.: A DNA microarray platform based on direct detection of rRNA for characterization of freshwater sediment-related prokaryotic communities. Appl. Environ. Microbiol. 72, 4829-4838 (2006)

21) Winter, C., Hein, T., Kavka, G., Mach, R.L., and Farnleitner, A.H.: Longitudinal changes in the bacterial community composition of the Danube River: a Whole-river approach. Appl. Environ. Microbiol. 73, 421-431 (2007)

22) Clesceri, L.S., Greenberg, A.E., and Eaton, A.D.: Standard methods for the examination of water and wastewater. 20th Ed., APHA, AWWA, WEF, Washington, DC (1998)

23) Pike, E.B., Carrington, E.G., and Ashburner, P.A.: An evaluation of procedures for enumerating bacteria in activated sludge. J. Appl. Microbiol., 35, 309-321 (1972)

24) Picard, C., Ponsonnet, C., Paget, E., Nesme, X., and Simonet, P.: Detection and enumeration of bacteria in soil by direct DNA extraction and polymerase chain reaction. Appl. Environ. Microbiol., 58, 2717-2722 (1992)

25) Iwamoto, T., Tani, K., Nakamura, K., Suzuki, Y., Kitagawa, M., Eguchi, M., and Nasu, M.: Monitoring impact of in situ biostimulation treatment on groundwater bacterial community by DGGE. FEMS Microbiol. Ecol., 32, 129-141 (2000)

26) Sei, K., Asano, K., Tateishi, N., Mori, K., Ike, M., Kohno, T., and Fujita, M.: Development of simple methods of DNA extraction from environmental samples for monitoring microbial community based on PCR. Jpn. J. Wat. Treat. Biol., 36, 193-204 (2000)

27) Allgaier, M. and Grossart, H.-P.: Diversity and Seasonal Dynamics of Actinobacteria Populations in Four Lakes in Northeastern Germany. Appl. Environ. Microbiol., 72, 3489-3497 (2006)

28) Sekiguchi, H., Watanabe, M., Nakahara, T., Xu, B., and Uchiyama, H.: Succession of bacterial community structure along the Changjiang River determined by denaturing gradient gel electrophoresis and clone library analysis. Appl. Environ. Microbiol., 68, 5142-5150 (2002)

29) Shannon, C.E. and Weaver, W.: The Mathematical Theory of Communication,

5th ed. University of Illinois Press, Urbana (1963)

30) Pesce, S., Fajon, C., Bardot, C., Bonnemoy, F., Portelli, C., and Bohatier, J.: Longitudinal changes in microbial planktonic communities of a French river in relation to pesticide and nutrient input. Aquat. Toxicol., 86, 352-360 (2008)

31) Cébron, A., Coci, M., Garnier, J., and Laanbroek, H.J.: Denaturing gradient gel electrophoretic analysis of ammonia-oxidizing bacterial community structure in the lower Seine River: impact of Paris wastewater effluents. Appl. Environ. Microbiol., 70, 6726-6737 (2004)

32) Iwane, T., Urase, T., and Yamamoto, K.: Possible impact of treated wastewater discharge on incidence of antibiotic resistant bacteria in river water. Water Sci. Technol., 43, 91-99 (2001)

33) Meyer, J.L.: The microbial loop in flowing waters. Microb. Ecol., 28, 195-199 (1994)

34) Olapade, O.A. and Leff, L.G.: Seasonal response of stream biofilm communities to dissolved organic matter and nutrient enrichments. Appl. Environ. Microbiol., 71, 2278-2287 (2005)