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# Microbial diversity in marine sediments from Sagami Bay and Tokyo Bay, Japan, as determined by 16S rRNA gene analysis

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**16S rDNA clone libraries were analysed to investigate the microbial diversity in marine sediments from Sagami Bay (stations SA, water depth of 1159 m, and SB, 1516 m) and Tokyo Bay (station TK, 43 m). A total of 197 clones was examined by amplified rDNA restriction analysis (ARDRA) using three four-base-specific restriction enzymes (*Hha*I, *Rsa*I and *Hae*III). In SA, 57 RFLP types were detected from 77 clones. In SB, 17 RFLP types were detected from 62 clones. In TK, 21 RFLP types were detected from 58 clones. The genotypic diversity among the three sampling sites was 0·958, 0·636 and 0·821, respectively, indicating that the microbial diversity of SA was higher than at the other two stations. At SA, the most abundant RFLP type constituted 10% of all clones. The samples from SB and TK had dominant RFLP types which constituted 60% and 38% of the total clone libraries, respectively. The community structure of SA included many single-type clones, which were found only once in the clone libraries. This structure contrasted with that of the other two stations. Thirty-seven clones were selected and sequenced according to dendrograms derived from ARDRA, to cover most of the microbial diversity in the clone libraries. No clones were identical to any of the known 16S rRNA sequences or to each other. All sequences had >84·8% similarity to rDNA sequences retrieved from the DNA databases. Sequenced clones fell into five major lineages of the domain *Bacteria*: the gamma, delta and epsilon *Proteobacteria*, Gram-positive bacteria and the division *Verrucomicrobia*. At SA, the *Verrucomicrobia* and the three subclasses of the *Proteobacteria* were found. Most clone sequences belonged to the gamma *Proteobacteria*. The high-GC Gram-positive bacteria and the gamma subclass of the *Proteobacteria* were common at both SB and TK. Although the depths of SB and TK were very different, the community diversity inferred from ARDRA and the taxonomic position of the dominant clones were similar. All clones belonging to the high-GC Gram-positive bacteria collected from both SB and TK fell into the same cluster and are regarded as members of an unknown actinomycete group. The clone compositions were different at each sampling site, and clones of the gamma *Proteobacteria* and high-GC Gram-positive bacteria were dominant.**

**Keywords:** microbial diversity, marine sediment, 16S rDNA, amplified rDNA restriction analysis (ARDRA), RFLP

## INTRODUCTION

During the past decade, the development of molecular techniques using nucleic acids has led to many new findings in studies of microbial ecology (Amann *et al.*, 1995). As a basic approach to clarifying microbial communities, 16S rDNAs are amplified by PCR from nucleic acids extracted from environmental samples, and then the PCR products are cloned and sequenced. This approach can avoid the limitation of the traditional culturing techniques for assessing the microbial diversity in natural environments and has been applied to water columns (Britschgi & Giovannoni, 1991; DeLong, 1992;

**Abbreviations:** ARDRA, amplified rDNA restriction analysis; NJ, neighbour-joining; RDP, Ribosomal Database Project.

The DDBJ accession numbers for the sequences reported in this paper are AB022607–AB022642.

DeLong *et al*., 1994; Fuhrman *et al*., 1992, 1993; Fuhrman & Davis, 1997; Giovannoni *et al*., 1990; Mullins *et al*., 1995), hot springs (Barns *et al*., 1994; Hugenholtz *et al*., 1998; Ward *et al*., 1990), soils (Kuske *et al*., 1997; Liesack & Stackebrandt, 1992; Ueda *et al*., 1995), sediments (Schleper *et al*., 1997; Wise *et al*., 1997), deep subsurface environments (Boivin-Jahns *et al*., 1995), hydrothermal vents (Moyer *et al*., 1995) and the gut of animals (McInerney *et al*., 1995; Ohkuma & Kudo, 1996).

Both sediment and soil probably represent some of the most complex microbial habitats on Earth. There may be several thousand species of bacteria in 1 g soil (Torsvik *et al*., 1990). To study the genetic diversity and to analyse the members of mixed microbial populations are two of the most important steps in microbial community studies. However, little research has been done on microbial diversity in marine sediments, and little information is currently available (Gray & Herwig, 1996).

We analysed 16S rDNA clone libraries to investigate the genetic diversity of microbial communities in marine sediments from Sagami Bay and Tokyo Bay. Clonal types were initially grouped on the basis of amplified rDNA restriction analysis (ARDRA). The 16S rDNA sequences from representatives of different RFLP groups were then determined and compared with those available from the DNA databases.

## METHODS

**Study areas.** Fig. 1 shows sampling sites. Sampling was done in February 1996 at two stations in Sagami Bay, SA (35° 4·0′ N, 139° 14·5′ E, water depth of 1159 m) and SB (35° 0·2′ N, 139° 20·5′ E, 1516 m), and at one station in Tokyo Bay, TK (35° 20·8′ N, 139° 47·1′ E, 43 m), by the R/V *Tansei-Maru* of the Ocean Research Institute, University of Tokyo. Tokyo Bay is located almost in the centre of Japan, and has an area of 1000 km². The mean depth is about 17 m. Industrial and domestic waste discharged from the large human population enters the bay mostly via four big rivers. Too much waste, aided by a low water exchange rate, has caused significant eutrophication (Coastal Oceanography Research Committee, The Oceanographical Society of Japan, 1985).

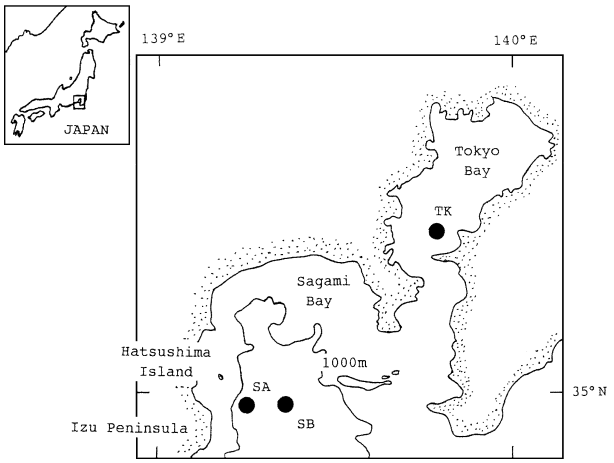


***Fig. 1.*** Map of sampling sites.

Sagami Bay is located in the south-west of Tokyo Bay. Water exchange is good, because this bay is well-connected to the Pacific Ocean and the Kuroshio Current flows strongly offshore. The maximum depth of the bay is more than 2000 m. The Western part of the bay is a steep fault escarpment flanking the north-eastern portion of Izu Peninsula. SA is near the bottom of the escarpment. A gentle slope continues from the SA area to the centre of the bay and, SB lies on this slope (Coastal Oceanography Research Committee, The Oceanographical Society of Japan, 1985).

**Sampling procedures and physical and chemical characteristics of marine sediments.** Sediment samples were collected with a multiple-core sampler (Rigosha). The sampler enabled us to take samples without any disturbance of surface sediments or contamination from surrounding seawater. Cores (82 mm in diameter) were aseptically sliced on board into 1 cm intervals with a stainless steel core cutter (Rigosha). Sliced samples were transferred into plastic bags, sealed with a thermo-sealer, and stored at −20 °C on board and at −80 °C in the laboratory until DNA extraction. The uppermost 1 cm sediment layer was used for further analysis. Sediment dry weights were measured after drying at 105 °C for 48 h. Total organic carbon and nitrogen were measured by a CHN analyser (NA1500NCS; Fisons Instruments).

Samples for direct counting were aseptically taken and placed into a plastic syringe (5 ml). One cubic centimetre of the sample was suspended in 9 ml filtered (0·22 μm) and autoclaved seawater and fixed with filtered formaldehyde (final concentration 4%) on board and stored at 4 °C. Sodium pyrophosphate solution was added to the samples (0·001 M final concentration), which were then sonicated and mixed (Velji & Albright, 1986). The total number of microorganisms was determined by counting cells stained with 4′, 6-diamidino-2-phenylindole (DAPI; 5 μg ml$^{-1}$ final concentration) (Schallenberg *et al*., 1989) under an epifluorescent microscope (BH-2; Olympus), as described by Porter & Feig (1980). At least 15 fields and more than 350 cells for total bacterial number were counted. Redox potential was measured with an ORP meter (RM-10P; TOA electronics). Table 1 lists characteristics of each site. Data are the mean of two or three experiments.

**DNA extraction and purification.** The DNA extraction method described by Ueda *et al*. (1995) was used with some modifications. Forty grams of sediment was mixed with an equal volume of 120 mM sodium phosphate buffer (pH 8·0) containing 5% SDS by vigorous shaking in a plastic bottle for 40 min at 40 °C. Then, 0·6 cm$^3$ of the mixture was transferred to 2·0 cm$^3$ plastic tubes and treated with seven cycles of freezing in liquid nitrogen and thawing in boiling water. The lysate was then purified by a SepaGene DNA extraction kit (Sankyo Junyaku). Electrophoresis on a 1% agarose gel (agarose 1600; Wako Pure Chemical Industries) was used to remove low-molecular-mass nucleic acids. DNA fragments larger than 9 kb were cut out and recovered with a GeneClean II kit (Bio 101) as recommended by the manufacturer.

**PCR amplification and cloning of 16S rDNA.** The primers used for amplification of bacterial 16S rDNA were 519f (5′-CAGCMGCCGCGGTAATWC-3′; positions 519–536 of *Escherichia coli* 16S rRNA numbering; Brosius *et al*., 1978) (Lane *et al*., 1985) and 1492r (5′-GGTTACCTTGTTACGACTT-3′; positions 1510–1492) (Eden *et al*., 1991). The PCR mixtures were prepared as described by Urakawa *et al*. (1997). DNA amplification was done in a DNA thermal cycler (PTC-

***Table 1.*** Sediment characteristics

| Station | $E_h$ (mV) | C (%) | N (%) | C:N (mol) | Moisture content (%) | $10^{-9}$ × No. of bacterial cells (dry g)$^{-1}$ |
|---|---|---|---|---|---|---|
| SA | 15 | 1·128 | 0·103 | 12·8 | 68·5 | 3·2 |
| SB | 84 | 2·350 | 0·266 | 10·3 | 88·8 | 1·2 |
| TK | −60 | 1·158 | 0·057 | 11·9 | 44·8 | ND |

ND, Not determined.

***Table 2.*** Summary of amplified rDNA restriction analysis

| Station | No. of clones | No. of RFLP types | RFLP type to clone number ratio | Genotypic diversity | Coverage | Number of restriction patterns when digested with: | | |
|---|---|---|---|---|---|---|---|---|
| | | | | | | *Hha*I | *Rsa*I | *Hae*III |
| SA | 77 | 57 | 0·74 | 0·958 | 0·45 | 20 | 39 | 49 |
| SB | 62 | 17 | 0·27 | 0·636 | 0·84 | 8 | 7 | 7 |
| TK | 58 | 21 | 0·36 | 0·821 | 0·74 | 10 | 12 | 9 |

100; MJ Research), with an initial denaturation for 2 min at 94 °C, 25 cycles of denaturation (2 min at 94 °C), annealing (1·5 min at 45 °C) and extension (2 min at 72 °C) and a final extension for 10 min at 72 °C to facilitate the TA cloning. Two microlitres of amplified DNA was examined by horizontal electrophoresis on 1% agarose gel in TAE buffer (40 mM Tris, 20 mM acetate, 2 mM EDTA). The amplified genes were cloned into plasmid vector pCRII of a TA cloning kit (Invitrogen). Blue–white selection was used for screening clones. Three hundred white clones were obtained in total. For reamplification of the cloned rDNA, clones were maintained on LB agar plates with ampicillin (50 μg ml$^{-1}$). Clones including correct-length inserts (approx. 900 bp) were checked by PCR using the M13 primers; 197 were obtained.

**Amplified rDNA restriction analysis.** To examine the RFLP patterns, 2 μl of a 1:10 dilution of the PCR product amplified by the M13 primers was reamplified by using the 519f and 1492r primers with the same PCR program as described above.

Six to eight microlitres of each PCR product was separately digested with three four-base-specific restriction enzymes (*Hha*I, *Rsa*I and *Hae*III) (Toyobo) at 37 °C for 120 min. Restricted DNAs were analysed by horizontal electrophoresis in 4% NuSieve 3:1 agarose gel (FMC BioProducts) on ice as previously described (Urakawa *et al.*, 1998a). The Super-ladder-low, 100 bp ladder (GenSure) was used as a DNA marker. Restriction fragments shorter than 99 bp were not considered in the analysis. A multi-lane screener program (Atto) was used for fragment pattern analysis. Genetic distances between pairs of clones were estimated from the proportion of shared restriction fragments by the method of Nei & Li (1979). Dendrograms were constructed from a distance matrix by using the unweighted pair group method using arithmetic means (Sneath & Sokal, 1973). Genetic diversity obtained by RFLP analysis was estimated by two different calculations, genotypic diversity (Nei, 1987) and coverage (Good, 1953; Mullins *et al.*, 1995; Rath *et al.*, 1998) (Table 2). Coverage ($C$) is derived from the equation $C = 1-(n1/N)$, where $n1$ is the number of clones that occurred only once (frequency = 1), and $N$ is the total number of clones examined.

**Determination of nucleotide sequences.** To determine the rDNA sequences, the PCR product amplified by the M13 primers was reamplified by using the 519f and 1492r primers with the same PCR program as described above. The PCR product was purified and concentrated with a Microcon-100 microconcentrator (Amicon).

Thirty-seven clones were selected according to the dendrograms derived from the results of ARDRA, and sequenced with a SequiTherm Long-Read Cycle Sequencing kit (Epicentre Technologies) and an automated sequencer (ALF DNA Sequencer; Amersham Pharmacia Biotech) as prescribed by Urakawa *et al.* (1998b). Primers for the sequencing described by Lane (1991) were used, and partial sequences corresponding to positions 600–1400 of *E. coli* were determined for each clone. These sequences were checked for chimeric artefacts by the CHECK_CHIMERA program of the Ribosomal Database Project (RDP) (Maidak *et al.*, 1997), and compared with similar rDNA sequences retrieved from the DNA databases, by using the FASTA search program in the DDBJ, the BLAST search program in the National Centre for Biotechnology Information (NCBI), and the RDP SIMILARITY_RANK program.

**Phylogenetic analysis.** Sequences were aligned by using the CLUSTAL W program (ver. 1.60) (Thompson *et al.*, 1994) and then realigned manually as previously described (Urakawa *et al.*, 1999). Nucleotide positions where there were ambiguous alignments and gaps were omitted from subsequent phylogenetic analysis. Neighbour-joining (NJ) analysis (Saitou & Nei, 1987) was also performed by using CLUSTAL W. The NJ tree was constructed from the distance matrix calculated by the algorithm of Kimura's two-parameter model (Kimura, 1980). Bootstrap confidence values were obtained with 1000 resamplings.

## RESULTS AND DISCUSSION

### Sample collection and DNA extraction

Nucleic acids were successfully extracted from the sediment samples without polyvinylpolypyrrolidone purification, which has been used for extracting DNA

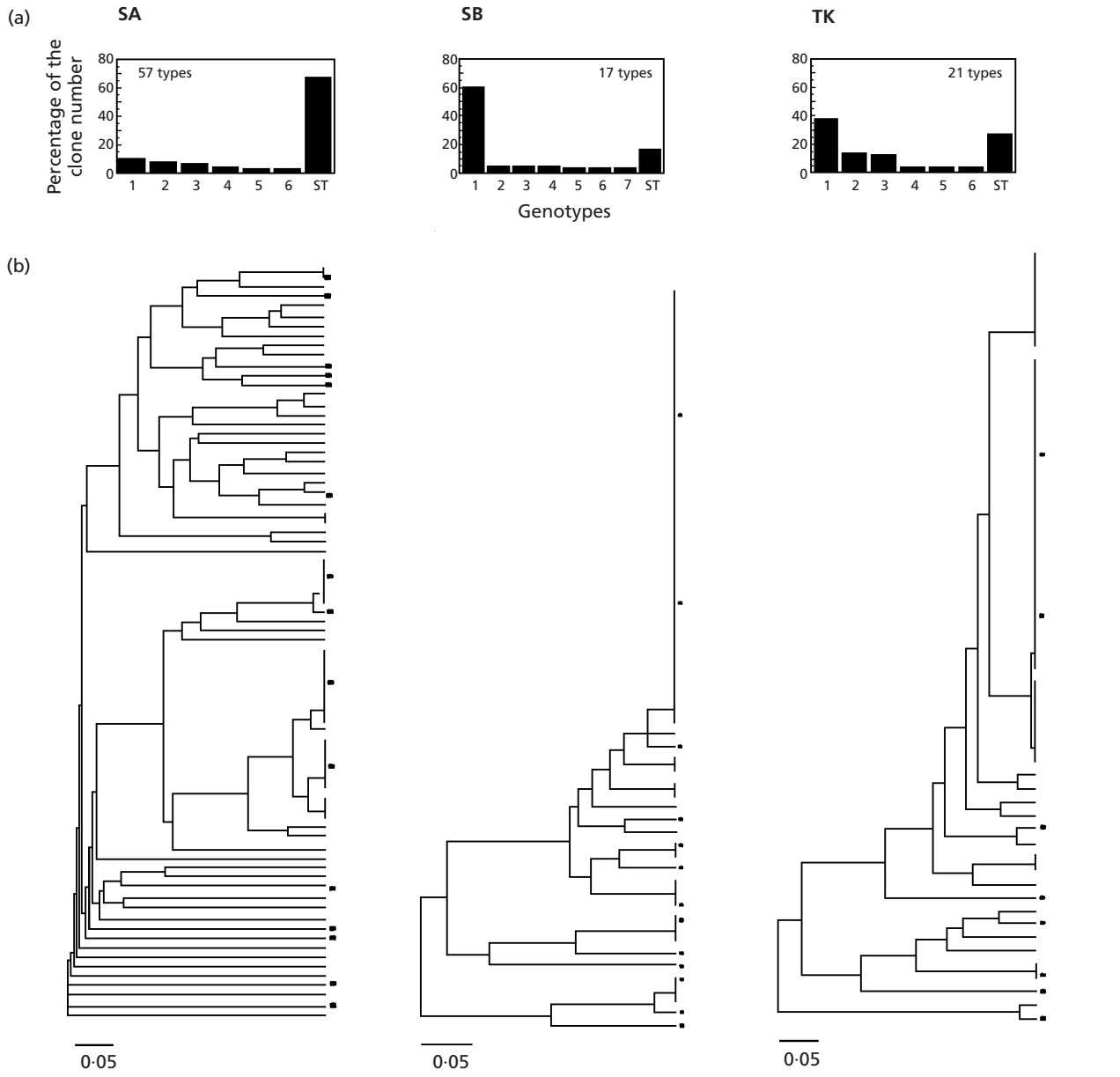


***Fig. 2.*** Relationship between 16S rDNA RFLP types and number of clones. (a) Genotype compositions. ST, single-type clones, which were found only once in the clone library. (b) Dendrograms constructed from ARDRA. Dots show the positions of sequenced clones.

from rich humic acid samples (Holben *et al*., 1988) for PCR amplification. The freeze–thaw technique used in this study was helpful in extracting DNA from marine sediment with little damage.

Recently, many investigators have raised questions about possible problems associated with PCR of the 16S rRNA gene for the phylogenetic analysis of microbial communities. As an increment of cycles of PCR could bias the clone composition obtained, we used 25 cycles in the PCR step to minimize bias (Sekiguchi *et al*., 1998; Suzuki & Giovannoni, 1996; Wilson & Blitchington, 1996). Primers for PCR amplification were selected to lessen a levelling effect of amplification (Suzuki & Giovannoni, 1996). Strong biases may be introduced by the copy number of 16S rRNA genes (Kerkhof & Speck, 1997) and the differential PCR amplification efficacy of DNA from heterogeneous templates (Chandler *et al*., 1997; Farrelly *et al*., 1995). These possible effects may mean that the proportions found in the clone libraries do not always represent the 16S rDNA proportions found in the original samples. However, the limitation of culture techniques (Amann *et al*., 1995) means that sequence-based phylogenetic techniques may provide a

***Table 3.*** Nearest neighbour of the 16S rDNA clones

| Clone | Length of sequence (bp) | Phylogenetic group | Accession no. of nearest neighbour | Nearest neighbour* | Similarity (%) | No. of clones included in the same restriction pattern |
|---|---|---|---|---|---|---|
| SA14 | 771 | *Chlamydia/ Verrucomicrobium* | X99392 | *Verrucomicrobium* sp. | 84·8 | 2 |
| SA15 | 582 | Gamma | M26631 | *Arhodomonas aquaeolei* | 88·4 | 1 |
| SA16 | 759 | Gamma | X74724 | *Vibrio splendidus* | 98·2 | 6 |
| SA28 | 821 | Gamma | X84980 | *Lucina pectinata* symbiont | 94·3 | 1 |
| SA41 | 848 | Gamma | X74724 | *Vibrio splendidus* | 97·5 | 8 |
| SA50 | 606 | Gamma | U77478 | *Riftia pachyptila* endosymbiont | 94·1 | 1 |
| SA51 | 764 | Gamma | L25711 | *Anodontia phillipiana* gill symbiont | 89·4 | 1 |
| SA56 | 809 | Delta | M34407 | *Desulfosarcina variabilis* | 91·7 | 1 |
| SA59 | 749 | Gamma | X74724 | *Vibrio splendidus* | 98·3 | 5 |
| SA63 | 634 | Epsilon | L35520 | *Alvinella pompejana* epibiont | 91·0 | 1 |
| SA68 | 680 | Gamma | U57919 | *Vibrio* sp. | 98·1 | 1 |
| SA71 | 709 | Delta | L35504 | *Nitrospina gracilis* | 87·0 | 1 |
| SA73 | 651 | Gamma | U57919 | *Vibrio* sp. | 88·6 | 1 |
| SA79 | 747 | Gamma | X74724 | *Vibrio splendidus* | 97·5 | 1 |
| SA82 | 822 | Delta | Y13672 | '*Desulfocapsa sulfoexigens*' | 90·2 | 1 |
| SB01 | 657 | Gram-positive | U09762 | *Clavibacter michiganensis* | 92·2 | 1 |
| SB06 | 654 | Gamma | AF030381 | *Pseudoalteromonas* sp. | 96·0 | 1 |
| SB09 | 672 | Gamma | L25707 | *Lucina floridana* gill symbiont | 90·4 | 1 |
| SB11 | 731 | Gram-positive | X77443 | *Microbacterium arborescens* | 92·0 | 1 |
| SB19 | 842 | Gram-positive | X77435 | *Clavibacter michiganensis* | 93·9 | 37 |
| SB20 | 728 | Gram-positive | X77443 | *Microbacterium arborescens* | 93·7 | 3 |
| SB21 | 712 | Gamma | D89792 | *Coxiella burnetii* | 88·9 | 1 |
| SB26 | 812 | Gram-positive | U09762 | *Clavibacter michiganensis* | 94·6 | 37 |
| SB33 | 644 | Gram-positive | AB015562 | Unidentified actinomycete | 93·7 | 3 |
| SB39 | 687 | Gram-positive | X77443 | *Microbacterium arborescens* | 94·3 | 3 |
| SB53 | 689 | Gram-positive | X77443 | *Microbacterium arborescens* | 94·6 | 1 |
| SB60 | 728 | Gram-positive | U09762 | *Clavibacter michiganensis* | 93·7 | 1 |
| SB66 | 733 | Gram-positive | X77443 | *Microbacterium arborescens* | 93·0 | 2 |
| TK01 | 669 | Gram-positive | AB015562 | Unidentified actinomycete | 94·0 | 22 |
| TK02 | 604 | Delta | M34407 | *Desulfosarcina variabilis* | 91·1 | 1 |
| TK12 | 748 | Gamma | L01575 | *Thyasira flexuosa* gill symbiont | 91·3 | 2 |
| TK19 | 844 | Gram-positive | X77435 | *Clavibacter michiganensis* | 93·7 | 1 |
| TK36 | 602 | Gram-positive | AB015562 | Unidentified actinomycete | 92·2 | 1 |
| TK75 | 752 | Gamma | U00006 | *Escherichia coli* | 94·2 | 1 |
| TK99 | 774 | Gamma | L01575 | *Thyasira flexuosa* gill symbiont | 89·3 | 1 |
| TK100 | 803 | Gram-positive | X77443 | *Microbacterium arborescens* | 93·2 | 22 |

* The closest matching sequence from a cultivated and characterized strain was identified using the FASTA search available in the DDBJ. In some cases, higher similarities were found with environmental rDNA clones, or uncharacterized strains.

less biased picture of community composition than would any single cultivation technique.

### Amplified rDNA restriction analysis

The summary of ARDRA is listed in Table 2. At SA, 57 RFLP types were detected from 77 clones. At SB, 17 RFLP types were detected from 62 clones. At TK, 21 RFLP types were detected from 58 clones. The genotypic diversity of SA was higher (0·958) than that at SB (0·636) and TK (0·821). Fig. 2(a) shows the relationships between RFLP types and number of clones. At SA, the most abundant RFLP type constituted 10% of all clones. In contrast, the most abundant RFLP types at the other two sites constituted 60% (SB) and 38% (TK) of the total clone libraries. Single-type clones, which occurred only once, were abundant at SA (66%), but were much less common at SB (16%) and TK (26%).

Moyer *et al*. (1994) examined a microbial mat community at a deep-sea hydrothermal vent by ARDRA.

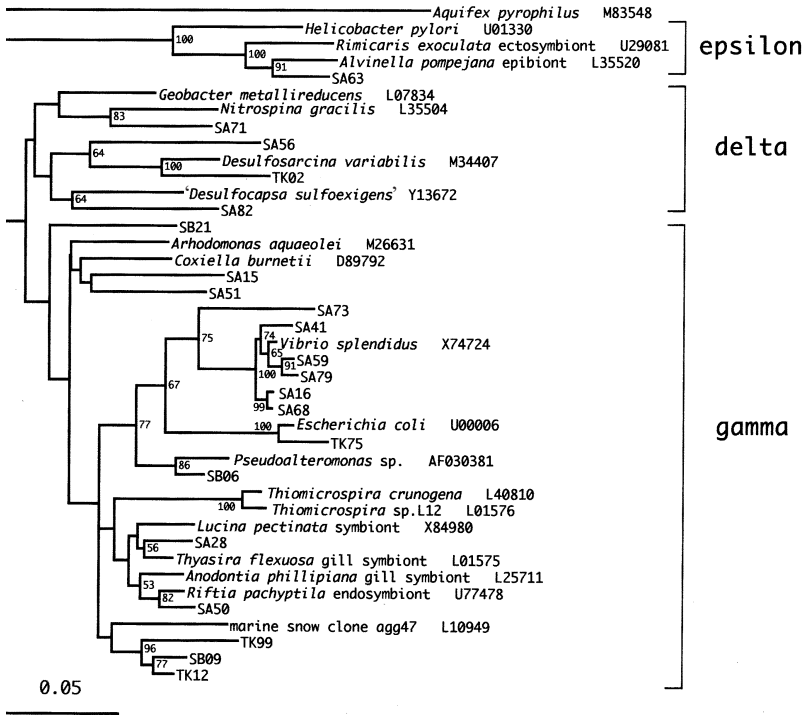


***Fig. 3.*** Phylogenetic tree of the *Proteobacteria*. The tree was constructed from a distance matrix by the NJ method. The analysis used 463 aligned positions. *Aquifex pyrophilus* was used as the outgroup. The scale bar represents the mean number of nucleotide substitutions per site. Percentages of bootstrap resampling (1000 resamplings) that support each topological element are indicated near the nodes (>50 %).

They obtained 12 RFLP types from 48 clones by using four four-base-specific restriction enzymes and found two dominant clone types. We made some calculations from their data; the genotypic diversity was 0·707, between those of SB and TK; the most abundant RFLP type in their data constituted 48 % of all clones. Mullins *et al.* (1995) estimated the coverage of an rDNA clone library of the Sargasso Sea to be 81 % and they also reported the coverage to be 68 % by analysis of SAR and ALO clone libraries reported by Schmidt *et al.* (1991), which were constructed from rDNAs collected from the open ocean. Coverage values from our SB and TK libraries were similar (Table 2), but the value from the SA library was much lower. Rath *et al.* (1998) also used coverage value to estimate the microbial diversity in marine snow samples as 5·3 %, the lowest value reported.

## Sequencing analysis of 16S rDNA clones

Thirty-seven clones were selected and sequenced according to dendrograms derived from ARDRA (Fig. 2b), so as to cover most of the microbial diversity in the clone libraries. We obtained 580–847 nucleotide sequences (mean size 702 bp) corresponding to approximately positions 520–1400 of *E. coli* numbering (Brosius *et al.*, 1978). One clone looked suspiciously chimeric when examined with the CHECK_CHIMERA program. One part was similar to Gram-positive bacteria, the other part to the gamma *Proteobacteria*. This sequence was not used for further phylogenetic analysis (data not shown).

Table 3 summarizes the clones sequenced. No clones were identical to any of the known 16S rRNA sequences from cultured organisms, environmental clones, or to each other in the library. All sequences had more than 84·8 % similarity to rDNA sequences in the DNA databases. Sequenced clones fell into five major lineages of the domain *Bacteria*: the gamma, delta and epsilon *Proteobacteria*, Gram-positive bacteria and the division *Verrucomicrobia* (Hedlund *et al.*, 1997) (Figs 3 and 4).

The community structure of SA contained many single-type clones, in contrast to the other two stations (Fig. 2a). As shown in Table 3, the *Verrucomicrobia* and the three subclasses of the *Proteobacteria* were found. The most frequently collected clones were the gamma *Proteobacteria*.

Community structures from SB and TK included dominant clones (Fig. 2a). 16S rDNA sequencing analysis showed that the high-GC Gram-positive bacteria and the gamma *Proteobacteria* were common to both (Table 3). Interestingly, in spite of very different depths at SB and TK, the dendrograms and the affiliations of sequenced clones were similar (Fig. 2b; Table 3). As shown in Fig. 4, clones belonging to the high-GC Gram-positive bacteria collected from both sites fell completely into the same cluster.

## Delta subclass of the *Proteobacteria*

Four clones (SA56, SA71, SA82 and TK02) were included in the delta *Proteobacteria* (Table 3). Phylogenetic analysis showed that SA56, SA82 and TK02 were related to the sulfate-reducing bacteria (Fig. 3). The redox potential at SB was higher than at the other stations (Table 1), and sulfate-reducing bacteria were found at

TK and SA, but not at SB. SA56 and TK02 were most similar (91–92% similarity) to *Desulfosarcina variabilis* (Table 3; Fig. 3). SA82 was most similar to '*Desulfocapsa sulfoexigens*' (90% similarity) by a FASTA search. SA71 was most similar to *Nitrospina gracilis* (87% similarity). This relationship is also supported by an 83% bootstrap confidence value on the phylogenetic tree (Fig. 3).

### Gamma subclass of the *Proteobacteria*

One frequently encountered group, consisting of five clones (SA16, 41, 59, 68 and 79) (Fig. 3), was apparently affiliated with the genus *Vibrio* (97–99% similarity) by 100% bootstrap confidence values. *Vibrio splendidus* was the closest species. SB06 was affiliated with *Pseudoalteromonas* sp. (96% similarity) (Table 3). TK75 was most similar (94·2%) to *E. coli* by a FASTA search. It was found only from Tokyo Bay (Table 3; Fig. 1).

SA15 was most similar to the sequence of *Arhodomonas aquaeolei* (the 16S rDNA sequence is registered as *A. oleiferhydrans* in the DNA databases), isolated from fluid of a petroleum production reservoir (Adkins *et al*., 1993), by a FASTA search (88·4% similarity) (Table 3). SA51 had higher similarity to an *Anodontia phillipiana* gill symbiont (89·4%) (Table 3). Phylogenetic analysis showed that this clone was closely related to *Coxiella burnetii* (Fig. 3). SB21 branched deeply within the gamma *Proteobacteria* lineage. The similarity values of SA15, SA51 and SB21 to other known species or clones were less than 90%. As phylogenetic relationships shown in Fig. 3 were not supported by high bootstrap values, it was difficult to speculate on the phenotypic features of these diverse clones.

Five clones (SA28, SA50, SB09, TK12 and TK99) showed a close relationship to sulfur-oxidizing bacterial endosymbionts by a FASTA search (Table 3). Phylogenetic analysis showed that two of these clones (SA28 and SA50) were clustered with the symbiotic thioautotrophs, which are known to be distributed across a broad range of host taxa in marine environments (Annelida, Mollusca, Pogonophora and Vestimentifera) (Durand & Gros, 1996), but are distinguished from *Thiomicrospira* species, which are free-living, obligately chemolithotrophic micro-organisms (Distel & Wood, 1992) (Fig. 3). However, the bootstrap support for most clades within the sulfur-oxidizing bacteria was low. SA28 was most similar (94·3%) to a sequence of *Lucina pectinata* endosymbiont (Table 3). SA50 was most similar (94·1%) to the endosymbiont of tube worm, *Riftia pachyptila* (Edwards & Nelson, 1991; Feldman *et al*., 1997).

The other three clones (SB09, TK12 and TK99) were clustered together with the marine snow clone agg47 reported by DeLong *et al*. (1993) (Fig. 3). Although detailed phylogenetic affiliation of this clone was not discussed in their report (DeLong *et al*., 1993), this clone has been treated as the sulfur-oxidizing bacterial group in the RDP. Thus these three clones are also included in this group. Gray & Herwig (1996) also found the sulfur-oxidizing bacterial group in coastal marine sediment. However, their sequence position is different from ours and cannot be compared.

### Epsilon subclass of the *Proteobacteria*

One clone, SA63, was affiliated with the epsilon *Proteobacteria* (Table 3; Fig. 3). It was most similar (91·0%) to an ectosymbiont of a polychaete, *Alvinella pompejana*, found at deep-sea hydrothermal vents, by a FASTA search (Cary *et al*., 1997; Haddad *et al*., 1995) (Table 3). This relationship was also supported on the phylogenetic tree by a 91% bootstrap confidence value (Fig. 3).

Moyer *et al*. (1995) reported the existence of epsilon *Proteobacteria* in a microbial mat community at Pele's Vent, a hydrothermal vent on Loihi Seamount, Hawaii. Recently, distribution of the *Alvinella pompejana* ectosymbiont, a member of the epsilon *Proteobacteria*, was examined at a hydrothermal vent and reported by Cary *et al*. (1997). Cary *et al*. (1997) concluded that the chemoautotrophic symbiotic gamma and epsilon *Proteobacteria* are likely to play an important role in the ecology of hydrothermal vent ecosystems.

*Calyptogena*-vestimentiferan tube worm communities were observed for roughly 7 km along the foot of the Hatsushima escarpment, Sagami Bay, between depths of 900 and 1200 m (Hashimoto *et al*., 1989; Kim *et al*., 1995). SA, at a depth of 1159 m, is close to this cold seep area. The total number of organisms at SA was higher than at SB (Table 1) and the redox potential of the sediment was lower than that at SB. Although it is difficult to conclude whether the symbiont-like clones are true members of this area, the community structure of SA might be affected by this area. Our results show that the symbiont-like gamma and epsilon *Proteobacteria* might be distributed in marine sediment as members of the community. Jones & Gardiner (1989) and Feldman *et al*. (1997) suggested that vestimentiferans acquire endosymbionts from the free-living bacterial community. Our result might contribute to studies of the evolutionary origin and acquisition of these endosymbionts. Little information is available about this symbiont-like group; further investigation is needed for clarifying its nature.

### *Verrucomicrobia*

One clone, SA14, was affiliated with the division *Verrucomicrobia* (Hedlund *et al*., 1997) (Table 3). This clone was most similar (84·8%) to *Verrucomicrobium* sp. strain VeSm13, found in the anoxic soil of flooded rice microcosms (Janssen *et al*., 1997). The phylogenetic tree also supports their close relationship with a 93% bootstrap confidence value (Fig. 4). Novitsky (1990) reported that marine sedimenting particles may influence sediment bacterial communities. This phylogenetic group was also found in marine sedimenting particles (Rath *et al*., 1998). From these reports, we have to assume two different habitats, marine snow and

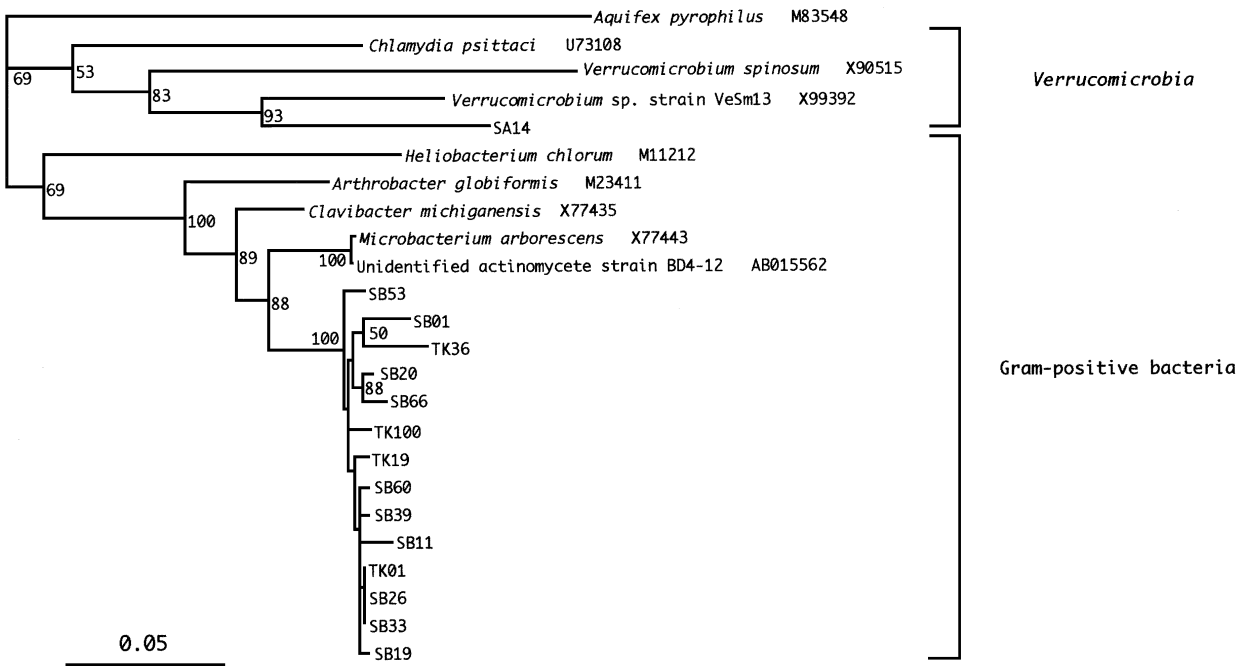


***Fig. 4.*** Phylogenetic tree of the Gram-positive bacteria and *Verrucomicrobia*. The tree was constructed from a distance matrix by the NJ method. The analysis used 561 aligned positions. *Aquifex pyrophilus* was used as the outgroup. The scale bar represents the mean number of nucleotide substitutions per site. Percentages of bootstrap resampling (1000 resamplings) that support each topological element are indicated near the nodes (>50%).

sediment, for this phylogenetic group in marine environments.

## Gram-positive bacteria

The most abundant sequences from SB and TK were the high-GC Gram-positive bacteria (Table 3). Fourteen clones showed close relationships to each other (93·0–99·8% similarity) and formed one large cluster supported by 100% bootstrap confidence values (Fig. 4). This group was dominant in the SB and TK clone libraries but was not found in SA. *Microbacterium arborescens*, *Clavibacter michiganensis* and an unidentified actinomycete were the closest species by a FASTA search (Table 3). It has been unclear for a long time whether the Gram-positive bacteria isolated from marine sediments are indigenous members of the microbial community in the sediments or whether they are carried from land by river flow. As shown in Fig. 4, new clones have been sequenced that are only distantly related to terrestrial isolates and this observation does indeed support the idea that this taxonomic group is separate from a previously known terrestrial group included in the family *Microbacteriaceae*. Moran *et al.* (1995) detected the presence of indigenous *Streptomyces* populations in marine sediments with the 16S rRNA probing technique. They concluded that true marine indigenous actinomycetes were present. Jensen & Fenical (1995) reported that 82% of the Gram-positive bacteria isolated from marine environments showed an obligate seawater requirement for growth. This result supports the conclusion of Moran *et al.* (1995). Quantitative analyses based on nucleic acid hybridization (Stahl & Amann, 1991), fatty acid analysis (Rajendran *et al.*, 1994) or quinone profile analysis (Hiraishi *et al.*, 1998) of these unknown Gram-positive bacteria are needed.

## Concluding remarks

The clone compositions were different at each sampling site. Sequenced clones fell into five major lineages of the domain *Bacteria*: the gamma, delta and epsilon *Proteobacteria*, Gram-positive bacteria and *Verrucomicrobia*. The gamma *Proteobacteria* and Gram-positive bacteria dominated in our clone libraries. Gray & Herwig (1996) detected six major lineages of the domain *Bacteria* and reported the dominance of the gamma *Proteobacteria* and Gram-positive bacteria in a marine sediment sample. This result also supports our data. However, alpha *Proteobacteria*, dominant or nearly dominant in various soil (Stackebrandt *et al.*, 1993; Zhou *et al.*, 1997) and seawater samples (Mullins *et al.*, 1995; Fuhrman & Davis, 1997), were not found.

Zhou *et al.* (1997) examined 43 16S rDNA clones from Siberian tundra soil, and found its diversity remarkable, because all clones had different RFLP patterns. We found that, in comparison with microbial communities observed in terrestrial soils (Kuske *et al.*, 1997; Ueda *et al.*, 1995; Zhou *et al.*, 1997), microbial communities in marine sediments (Gray & Herwig, 1996) were less

variable and simple at the phylum or class level, because marine sediments are covered with seawater. Such stability is also observed in microbial communities in the open ocean (Mullins *et al*., 1995). Further phylogenetic studies and functional analyses are required for understanding the microbial diversity and community structure in marine sediments. Other kinds of studies are necessarily required to supplement phylogenetic studies (Stahl & Amann, 1991; Rajendran *et al*., 1994; Hiraishi *et al*., 1998).

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