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Phylogenetic Compositions of Bacterioplankton from Two California Estuaries Compared by Denaturing Gradient Gel Electrophoresis of 16S rDNA Fragments

ALISON E. MURRAY,[1,2]† JAMES T. HOLLIBAUGH,[1]* AND CRISTIÁN ORREGO[2]

Center for Environmental Studies, San Francisco State University, Tiburon, California 94920,[1] and Conservation Genetics Laboratory, Biology Department, San Francisco State University, San Francisco, California 94132[2]

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The phylogenetic compositions of bacterioplankton assemblages from San Francisco Bay and Tomales Bay, Calif., differed substantially when analyzed by PCR-denaturing gradient gel electrophoresis; these differences are consistent with the results of previous studies demonstrating differences in their metabolic capabilities. PCR-denaturing gradient gel electrophoresis analysis of complex microbial assemblages was sensitive and reliable, and the results were reproducible as shown by experiments with constructed and naturally occurring assemblages.

San Francisco Bay and Tomales Bay are estuaries on the central coast of California (Fig. 1). Although they are close to each other and experience the same climatology, they are quite different physically and biologically because of differences in the relative amounts of fresh water inflow (high in San Francisco Bay), turbidity (high in San Francisco Bay), and primary production (low in San Francisco Bay) (2, 3, 10, 27). These factors combine to make detrital material delivered by river inflow an important source of organic C to San Francisco Bay (13), while phytoplankton production is the dominant source of organic C in Tomales Bay (27).

Previous studies of the metabolic fate of thymidine and the use of a variety of carbon sources (8, 9, 11) have demonstrated clear differences in bacterioplankton metabolism between San Francisco Bay and Tomales Bay. Tomales Bay bacterioplankton predominately incorporate thymidine into DNA while San Francisco Bay bacterioplankton metabolize it. Bacteria from San Francisco Bay communities were able to grow on most of the substrates in BIOLOG GN plates, while Tomales Bay bacterioplankton were more fastidious. These differences have been hypothesized to be due to differences in the species compositions of these communities, which in turn are driven by differences in the kinds of organic substrates available to the bacterioplankton communities (9). Alternatively, the possibilities that species compositions are the same and that metabolic differences are due to differences in carbon sources are plausible.

To test the hypothesis that the bacterioplankton compositions are different we analyzed the phylogenetic diversity of these bacterioplankton assemblages by comparison of PCR-amplified rRNA phylotype profiles produced by denaturing gradient gel electrophoresis (DGGE) (7, 17–20). Experiments with constructed and naturally occurring assemblages addressed the sensitivity and reliability of this methodological approach. This approach was then used to examine spatial and temporal variations in the composition of bacterioplankton assemblages within and between Tomales Bay and San Francisco Bay.

* Corresponding author.
† Present address: Biology Department, University of California, Santa Barbara, CA 93106.

Reference DNAs. We used bacterial cultivars obtained from surface seawater collected in Tomales Bay on 30 November 1993 and in San Francisco Bay on 13 December 1993 (Fig. 1) and eight bacterial genomic DNAs with 16S rRNA gene sequences that have been characterized: *Agrobacterium tumefaciens*, *Alcaligenes eutrophus*, *Desulfovibrio desulfuricans*, *Pseudomonas testosteroni*, *Chlorobium limicola*, *Deinococcus radiodurans*, *Streptomyces coelicolor*, and *Thermotoga maritima* (graciously donated by Norman Pace and Ester Angert, Indiana University, Bloomington, Ind.) as reference DNA for constructed assemblages and gel mobility standards. Cultivars were screened on the basis of morphology, color, growth rate, and production of a single, unique band in PCR-DGGE with primers GC358f and 517r and assigned strain designations as listed in the legend to Fig. 2.

DNA extraction and PCR. Cultivar and bacterioplankton DNA was extracted and purified by standard methods as described in detail elsewhere (15). The DNA was rehydrated in minimal amounts of Tris-EDTA buffer (100 μl for estuarine cultivars and 30 μl for bacterioplankton extracts) to ensure concentrated extracts. The DNA concentration was estimated by the Hoechst dye assay (22).

Variable region 3 of the 16S rRNA gene (21) was amplified by PCR (25) with primer sequences complementary to positions 341 to 358 (primer 358f; eubacterial) and positions 517 to 534 (primer 517r; universal) corresponding to *Escherichia coli* 16S rRNA numbering (17). A 40-bp GC clamp (18, 26) was added to the 358f primer. The primer sequences were 5′-CCTACGGGAGGCAGCAG-3′ for 358f, 5′-ATTACCGC GGCTGCTGG-3′ for 517r, and 5′-CGCCCGCCGCGCCCC GCGCCCGTCCCGCCGCCCCCGCCCCCCTACGG GAGGCAGCAG-3′ for GC358f. PCR conditions including the hot start (5) and touchdown for primer annealing (4) were similar to those used by Muyzer et al. (17). Each 50-μl reaction mixture consisted of 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 or 2.5 mM $MgCl_2$, 0.001% gelatin, 0.25 U of *Thermus aquaticus* DNA polymerase (Perkin Elmer Ampli*Taq* DNA polymerase LD), a 0.5 μM concentration of each primer, 0.75 mM deoxynucleoside triphosphate mixture, and 10 to 20 ng of template DNA. Filter and extraction blanks were analyzed along with each set of samples. The thermal cycling program was as follows: initial denaturation at 94°C for 3 min, denatur-

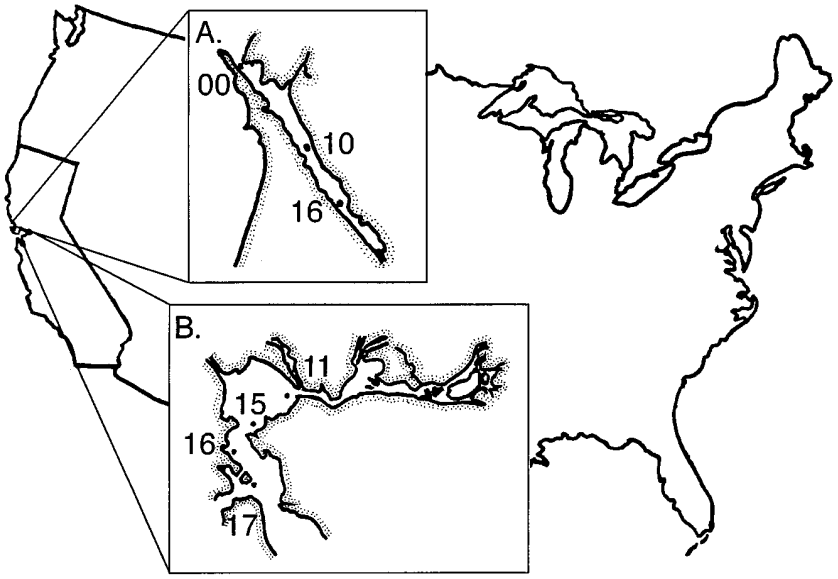

FIG. 1. Map of California estuaries sampled in this study (not to scale). (A) Tomales Bay sampling stations, previously described by Smith et al. (27), were sampled on 30 November 1993 and 2 May 1994; (B) San Francisco Bay sampling stations, described in reference 2, were sampled on 13 December 1993 and 16 May 1994.

ation at 94°C for 30 s, and touchdown primer annealing from 65 to 55°C for 30 s (annealing temperature decreased 1°C for each cycle for the first 10 cycles to a final temperature of 55°C), annealing at 55°C for 30 s for the next 20 cycles, and then primer extension at 72°C for 30 s. The reaction mixture was run for a total of 30 cycles, followed by a final extension for 5 min at 72°C. We confirmed amplification of the targeted region by restriction enzyme digestion of fragments amplified from reference DNA. Analysis of the PCR products revealed variability in the length of the amplified fragment. The most common fragment sizes were 169, 174, 190, and 194 bp (excluding the clamp sequence).

DGGE. Acrylamide stock solutions (6.5% acrylamide and 37.5:1 ratio of acrylamide-bisacrylamide) containing no denaturant or an 80% concentration of the denaturant (100% concentration of the denaturant is 7 M urea–40% deionized formamide) were used to prepare gels with a 40 to 70% denaturing gradient. All gels were run for 5 h in 1× TAE buffer (40 mM Tris base, 20 mM sodium acetate, 1 mM EDTA) at 60°C and a constant voltage of 10 V/cm in a CBS Scientific DGGE system. Approximately 40 ng of PCR product was used for DGGE analysis of reference DNA. The products of three or four PCR reactions were combined for bacterioplankton samples (total of 600 to 900 ng of DNA loaded) to maximize the probability of detecting rare sequences. Following Muyzer et al. (16), we refer to each band detected as a phylotype.

PCR-DGGE analysis of constructed assemblages. To address questions regarding the sensitivity and reliability of multitemplate DNA amplification and PCR-DGGE analysis, two artificial assemblages, A and B (18 constituents each), were constructed by mixing reference DNAs (see the legend to Fig. 2). Assemblage A was constructed with 5 ng of each constituent DNA (total, 90 ng of DNA). Assemblage B contained DNA from five of the estuarine cultivars at 50 ng each and DNA from the other 13 constituents at 5 ng each (total, 315 ng of DNA). The reference DNAs were amplified individually and run in the same gel for direct comparison of band migration. Immediately prior to DGGE analysis, replicates from each assemblage were heated to 100°C for 5 min and cooled to 50°C over a 30-min period to increase the probability of heteroduplex formation (24).

Thirteen and 15 of the 18 bands expected were detected in assemblages A and B, respectively. The three DNAs not detected in either assemblage (TB10B, *Alcaligenes eutrophus*, and *Deinococcus radiodurans*) may have comigrated with other

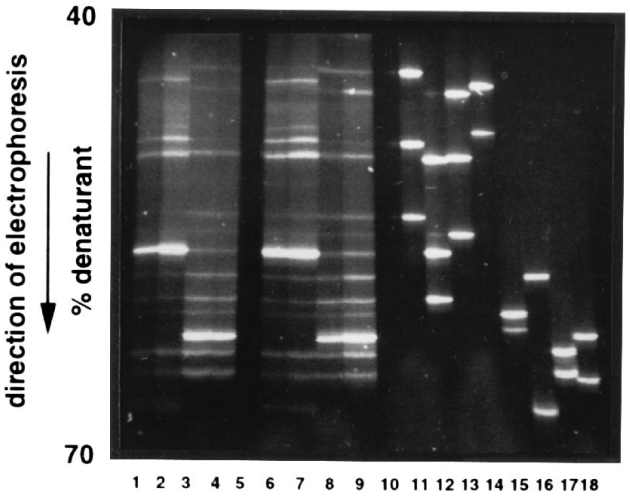

FIG. 2. PCR-DGGE analysis of constructed assemblages. Lanes: 1 to 4, samples treated to optimize potential heteroduplex formation; 5, blank; 6 to 9, samples analyzed by normal procedure; 10, blank; 11 to 18, standards. Assemblage A (lanes 3, 4, 8, and 9) was constructed by combining 5 ng of genomic DNA from each of the following: isolates SF00B, SFE1A, SFE1C, SFE1F, SFE2A, SFE2B, SFE2C, SFE2E, SFE3C, TB00D, TB00E, and TB10B; *Agrobacterium tumifaciens*; *Alcaligenes eutrophus*; *Chlorobium limicola*; *Deinococcus radiodurans*; *Desulfovibrio desulfuricans*; and *Streptomyces coelicolor*. Assemblage B (lanes 1, 2, 6, and 7) was constructed by combining 50 ng of genomic DNA (each) from isolates SFE1F, SFE2C, SFE2E, TB00D, and TB00E with 5 ng of genomic DNA (each) from isolates SF00B, SFE1A, SFE1C, SFE1F, SFE2A, SFE2B, SFE3C, and TB10B; *A. tumifaciens*; *A. eutrophus*; *C. limnicola*; *Deinococcus radiodurans*; *Desulfovibrio desulfuricans*; and *Pseudomonas testosteroni*. PCR products from amplification of the 16S rDNA variable region 3 of individual constituents were run as standards in lanes 11 to 18 for comparison. These PCR products were loaded in combinations for DGGE analysis as follows, from lower to higher mobility: SF00B, TB00D, and SFE1A (lane 11); TB00E, SFE1F, and SFE1C (lane 12); SFE2A, TB10B, and SFE2B (lane 13); SFE2C and SFE2E (lane 14); *A. tumifaciens* and *A. eutrophus* (lane 15); SFE3C and *S. coelicolor* (lane 16); *Desulfovibrio desulfuricans* and *C. limicola* (lane 17); and *P. testosteroni* and *Deinococcus radiodurans* (lane 18).

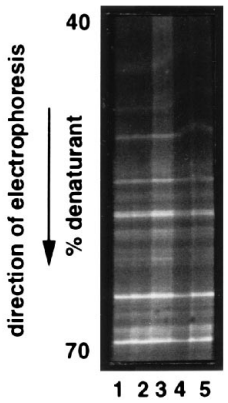

FIG. 3. Phylotype profile analysis of five replicate samples from San Francisco Bay station 11 collected on 13 December 1993.

bands, since all three had mobilities similar to those of other DNAs (Fig. 3, lanes 11 to 18). The significance of this error will increase with the complexity of the bacterial assemblage and is exacerbated by fragment length variation. The other two bands (SFE2C and SFE2E) not detected in assemblage A were detected in assemblage B in which their concentrations in the template mixture were greater.

Assemblage A contained similar concentrations of DNA in each DGGE band (Fig. 2, lanes 3, 4, 8, and 9). Bands representing constituents amplified with high initial template DNA concentrations (50 ng, assemblage B) stained more intensely than constituents with 5 ng of template DNA (Fig. 2, lanes 1, 2, 6, and 7). While this pattern suggests a relationship between band intensity and the relative abundance of DNA from that phylotype in the template mixture, variation in 16S rRNA gene copy numbers (6), PCR biases (23, 28), or comigration of bands from different phylotypes could also cause variation in band intensity (for example, the relatively strong signal of *P. testosteroni* in assemblage A). Muyzer et al. (17) detected all of the bands expected from a five-member constructed assemblage, although there was some variation in band intensity also.

Banding patterns of samples treated to enhance heteroduplex formation (Fig. 2, lanes 1 to 4) were identical to those of samples treated normally (lanes 6 to 9). One band in assemblage A (lanes 8 and 9) had a lower mobility than any reference DNAs (Fig. 2, lanes 8 and 9) and may have been either a heteroduplex or a contaminant. These experiments indicate that heteroduplex formation does not significantly interfere with PCR-DGGE analysis of complex communities.

We easily detected template DNA present at 1.6% of the total template DNA in assemblage B (Fig. 2), in accordance with the finding in the previous study by Muyzer et al. (17).

Analysis of natural bacterioplankton assemblages. Surface seawater was collected by boat at stations in Tomales Bay and San Francisco Bay (Fig. 1). Eight replicate subsamples (300 ml each) of the particulate material passing through a Whatman GF/C filter (nominal pore size, 1.2 μm; <20-kPa vacuum) were collected on 0.22-μm-pore-size Durapore filters (25-mm diameter; Millipore Corporation; 100-kPa vacuum). Each filter was placed in a 1.5-ml microcentrifuge tube with lysis buffer and either frozen immediately at −20°C or archived at −80°C.

Five replicate subsamples from San Francisco Bay station 11 (13 December 1993) were processed, amplified independently, and then analyzed on the same DGGE gel. The phylotype profiles of the replicate samples were identical and showed 26 phylotypes with the same relative amounts of DNA per band (Fig. 3), indicating that assemblages can be compared reliably since there is no discrepancy in the banding pattern between replicates.

Spatial and temporal phylotypic variation. Phylotype profiles of 14 samples from both bays collected in December 1993 and May 1994 (Fig. 4) were compared. The gel was stained with Tangerine nucleic acid stain (Molecular Probes), and then the staining intensities and band locations were determined with an FMBIO-100 gel documentation system (Hitachi). The total band counts (phylotype richness) and relative band mobilities were determined, and a graphical reproduction of the gel was prepared from the scanned image (Fig. 4). The sample for which the results are shown in Fig. 3 was also run on this gel and is shown in column 13.

Our analyses indicate high degrees of complexity in these samples: 15 to 31 phylotypes per profile, which we believe to be a lower limit of richness. The total band counts (phylotype richness) of the assemblages that we sampled were fairly consistent within an estuary and differed between estuaries: 22 to 31, mean 26, phylotypes per San Francisco Bay sample versus 15 to 24, mean 20, per Tomales Bay sample.

Five phylotypes found in all samples may represent common coastal bacterioplankton entrained by estuarine circulation. Three of these ubiquitous phylotypes are the most intense bands in our samples and may represent a numerically dominant lower taxon (i.e., species) or a higher taxon (i.e., family) represented by more than one subdominant lower taxon. Analysis of the nucleotide sequence of the ubiquitous band with highest mobility (Fig. 4, row 59) indicated high similarity (0.862 by the Similarity Rank program of the Ribosomal Database Project [14a]) to sequences in the SAR 11 cluster of α-proteobacteria.

The phylotype profiles of the samples from the two bays were compared by Sorenson's index, $C_s = 2j/(a+b)$, a pairwise similarity coefficient (14), where j is the number of sequences found common to both sites, a is the number of sequences at site A, and b is the number of sequences at Site B. A C_s value of 0 indicates that the samples are completely different, and a C_s value of 1 indicates that the samples are identical. Comparison of samples from midbay stations, Tomales Bay station 10 and San Francisco Bay station 15, gave a C_s value of 0.58 for December and 0.49 for May. There was greater similarity through time at given stations in both bays (Tables 1 and 2), in agreement with the findings of previous studies (9, 12) which have shown that the metabolic properties of bacterioplankton communities differ dramatically between these estuaries but that they are fairly consistent within each estuary.

The similarity of samples between adjacent stations was greater than the similarity with samples from distant stations (Tables 1 and 2), suggesting replacement of phylotypes along the environmental gradient. This relationship was strongest for the December samples, though salinity distributions were sim-

TABLE 1. Similarity (C_s) matrix for Tomales Bay bacterioplankton phylotype profiles

Station no.	C_s value for station no. indicated[a]		
	0	10	16
0	**0.75**	0.51	0.59
10	0.68	**0.56**	0.74
16	0.57	0.74	**0.6**

[a] The values in the lower left are for the December samples, and the values in the upper right are for the May samples. The boldfaced values along the diagonal are the similarity between December and May samples for each station.

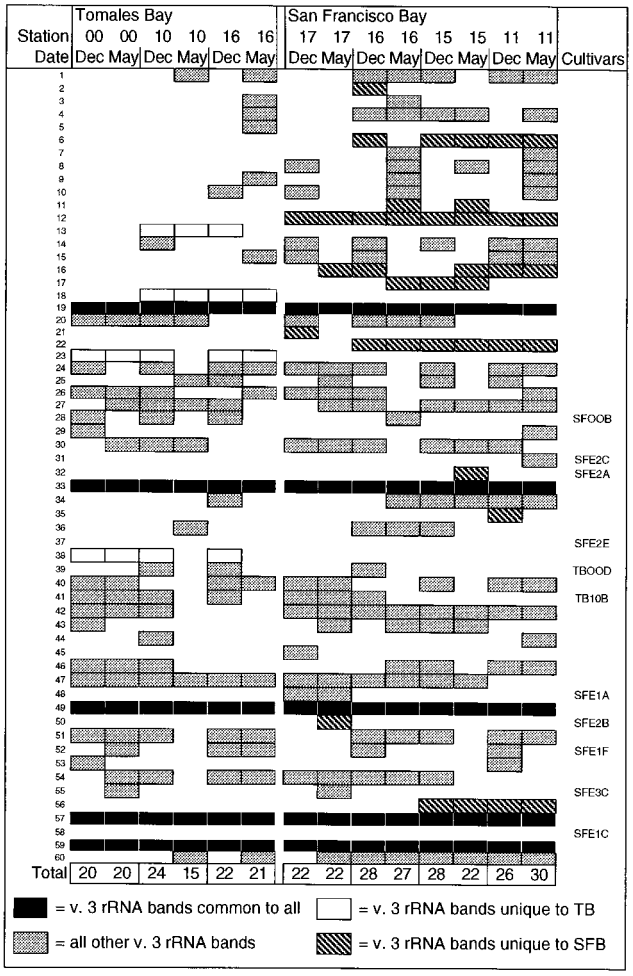

FIG. 4. Graphic representation of DGGE profiles of PCR products from bacterioplankton DNA collected from Tomales Bay (TB) and San Francisco Bay (SFB) as indicated in the text. Cultivar strain designations listed on the right indicate the mobilities of the PCR products of these cultivars in this gel. v. 3, variable region 3.

ilar during both sampling periods. Hollibaugh (9) reported that the degree to which San Francisco Bay populations (where the salinity gradient is greatest) metabolize thymidine also varied with the salinity gradient. Chin-Leo and Benner (1) have reported a similar phenomenon in the Mississippi River plume. Differences in the quality of organic matter along the gradient may control community composition and thus the shift in phylotype profiles; however, mixing of different assemblages may also contribute to the observed gradient.

Our analysis of the phylogenetic compositions of San Francisco Bay and Tomales Bay bacterioplankton supports the hypothesis that observed differences in metabolic capabilities are reflected in the compositions of bacterioplankton communities. However, since five of the phylotypes appear to be common to both bays, other factors such as the distribution of active cells among phylotypes or the expression of specific metabolic characteristics by metabolically plastic species must also be important. Phylotype profile analysis has enabled us to quantitatively address the extent of phylogenetic variability in estuarine bacterioplankton assemblages and to address the ecological question of whether observed metabolic differences are due to the physiological state of the same microbial assemblages or to differences in the phylogenetic compositions.

TABLE 2. Similarity (C_s) matrix for San Francisco Bay bacterioplankton phylotype profiles

Station no.	C_s value for station no. indicated[a]			
	17	16	15	11
17	**0.68**	0.45	0.55	0.59
16	0.64	**0.58**	0.65	0.66
15	0.56	0.75	**0.72**	0.62
11	0.48	0.66	0.77	**0.75**

[a] The values in the lower left are for the December samples, and the values in the upper right are for the May samples. The boldfaced values along the diagonal are the similarity between the December and May samples for each station.

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