Diversity and Abundance of Uncultured Cytophaga-Like Bacteria in the Delaware Estuary

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The Cytophaga-Flavobacterium group is known to be abundant in aquatic ecosystems and to have a potentially unique role in the utilization of organic material. However, relatively little is known about the diversity and abundance of uncultured members of this bacterial group, in part because they are underrepresented in clone libraries of 16S rRNA genes. To circumvent a suspected bias in PCR, a primer set was designed to amplify 16S rRNA genes from the Cytophaga-Flavobacterium group and was used to construct a library of these genes from the Delaware Estuary. This library had several novel Cytophaga-like 16S rRNA genes, of which about 40% could be grouped together into two clusters (DE clusters 1 and 2) defined by sequences initially observed only in the Delaware library; the other 16S rRNA genes were classified into an additional four clades containing sequences from other environments. An oligonucleotide probe was designed for the cluster with the most clones (DE cluster 2) and was used in fluorescence in situ hybridization assays. Bacteria in DE cluster 2 accounted for about 10% of the total prokaryotic abundance in the Delaware Estuary and in a depth profile of the Chukchi Sea (Arctic Ocean). The presence of DE cluster 2 in the Arctic Ocean was confirmed by results from 16S rRNA clone libraries. The contribution of this cluster to the total bacterial biomass is probably larger than is indicated by the abundance of its members, because the average cell volume of bacteria in DE cluster 2 was larger than those of other bacterial groups and prokaryotes in the Delaware Estuary and Chukchi Sea. DE cluster 2 may be one of the more abundant bacterial groups in the Delaware Estuary and possibly other marine environments.

Identifying which groups of heterotrophic bacteria are abundant in aquatic ecosystems is an important step in determining which microbes dominate fluxes of dissolved organic material (DOM) and other aspects of carbon cycling. The abundance and biomass of bacteria in a taxonomic group are likely to be indicative of that group’s relative importance in DOM fluxes. For example, in the Delaware Estuary (the body of water comprising the Delaware River and the Delaware Bay), the abundance of various bacterial groups explains about 50% of the variation in their contributions to bacterial production (thymidine and leucine incorporation) (10), which is one index of DOM uptake; rare bacterial groups (<10% of total abundance) did not appear to contribute substantially to production (<10% of total production). These data suggest that, although rare groups may be important in using some DOM components and in mediating other biogeochemical processes, the abundant heterotrophic bacterial groups dominate DOM uptake and carbon cycle processes mediated by the microbial loop in aquatic environments.

Of over 35 bacterial divisions found in the biosphere (25), only a few appear to be abundant in aquatic ecosystems. Proteobacteria and the Cytophaga-Flavobacterium group, which is a complex group in the Bacteroidetes division, usually dominate heterotrophic bacterial communities in oxic surface waters of lakes, rivers, and the oceans (20, 21). We probably know the most about the proteobacteria, in particular one clade, SAR11, in the alpha-proteobacterial subdivision (18). The SAR11 clade dominates 16S rRNA clone libraries constructed from bacterial assemblages from several marine habitats around the world (20) and appears to be quite abundant in the oceans, according to RNA probing and fluorescence in situ hybridization (FISH) assays (32).

Members of the Cytophaga-Flavobacterium group are also abundant in the oceans, based on FISH results (21, 27). The FISH assays for Cytophaga-Flavobacterium spp. usually rely on a single oligonucleotide probe (CF319a) (3), although a few other probes are now available (33, 44). The CF319a probe binds to several diverse bacteria in the Bacteroidetes division, ranging from aerobic Cytophaga spp. to anaerobic Bacteroides spp. (33, 44), but it also does not recognize several subgroups of Cytophaga-Flavobacterium organisms which are potentially common in aquatic ecosystems (33, 44). Previous studies have suggested alternatives to the CF319a probe (33, 44), but there has been less work on devising probes for subgroups or subdivisions of the Bacteroidetes division. Eilers et al. (16) found that one lineage of Cytophaga-like bacteria (the Cytophaga marinoflava-Cytophaga latercula lineage) made up 19% of all Cytophaga-like bacteria recognized by the CF319a probe and about 5% of all prokaryotes in the North Sea between April and September. Other than the information in that report, little is known about the number and relative abundance of subgroups of Cytophaga-Flavobacterium organisms in natural aquatic ecosystems.

The paucity of information about Cytophaga-Flavobacterium subgroups is partly due to the relatively low number of 16S rRNA sequences available for these microbes. The Cytophaga-Flavobacterium group appears to be underrepresented in the 16S rRNA database. For example, the latest version of the
Ribosomal Database Project (December 2002) has over 27,000 sequences for the Proteobacteria division, of which, nearly 8,000 are in the alpha subdivision (30). In contrast, there are only about 175 and 1,200 sequences in the Cytophaga genus and Flavobacteria class, respectively, out of about 4,300 sequences for the entire Bacteroidetes division. Likewise, in contrast to several published analyses of whole genomes from Proteobacteria, only one from the Bacteroidetes division has been published (46), although several others are in the process of being sequenced and analyzed (http://www.tigr.org/tdb/mdb/mbiprogress.html). Relatively few Cytophaga-like 16S rRNA sequences are available, probably because these microbes are often underrepresented in clone libraries of 16S rRNA genes even when Cytophaga-Flavobacterium organisms are abundant in natural microbial assemblages according to FISH (9). The bias against the Cytophaga-Flavobacterium group is partially explained by mismatches with the general bacterial PCR primers (41), although other sources of bias are likely (9).

The purpose of this study was to explore the diversity and abundance of Cytophaga-Flavobacterium subgroups in the Delaware Estuary. Estuaries like the Delaware Estuary are interesting aquatic environments for the examination of bacterial diversity, because the major bacterial groups vary greatly within the estuary and over time in response to salinity and other factors (12, 13). We found a few subgroups of Cytophaga-Flavobacterium spp. that were abundant in the Delaware Estuary and one subgroup that was also present in the Arctic Ocean. These Cytophaga-Flavobacterium subgroups may join SAR11 as potential candidates for the most abundant bacterial group in the oceans.

### MATERIALS AND METHODS

#### Library construction.

The bacterial size fraction (diameter, <3.0 μm) was collected at a station 10 km from Cape Henlopen within the Delaware Estuary in March 2001. About 85% of the bacterioplankton was in this size fraction as determined by direct-count epifluorescence microscopy (35). To isolate bacterioplankton DNA, the <3.0-μm size fraction (ca. 6 liters) was filtered onto Millipore Durapore filters (pore size, 0.22 μm; type GVWP) which were stored frozen at ~−80°C in a storage buffer (19). Frozen filters were thawed, and cells were lysed by using sodium dodecyl sulfate and proteinase K for 3 h. The lysate was extracted sequentially with phenol-chloroform and chloroform. The DNA was precipitated with sodium acetate and ethanol and was collected by centrifugation. The DNA was extracted sequentially with phenol-chloroform and chloroform. The lysate was precipitated with sodium dodecyl sulfate and proteinase K for 3 h. The lysate was precipitated with sodium acetate and ethanol and was collected by centrifugation.

Two libraries of 16S rRNA genes were constructed with DNA isolated from the Delaware Estuary. A general bacterial library was constructed from amplifications of a PCR using the universal bacterial primers EubB and EubA (Table 1). The second library targeted 16S rRNA genes from Cytophaga-like bacteria (Cytophaga-Flavobacterium [CF] library). The library consisted of amplicons of the PCR using a new primer (CF316) designed during this study (Table 1) and the universal bacterial primer EubA. The PCR conditions were similar to those used for the EubB and EubA primer set, except that the MgCl2 concentration was lower, 1.2 mM rather than 2 mM, to reduce amplification of nonspecific products (see Results). The added volumes (and stock concentrations) of deoxynucleoside triphosphates (10 mM), MgCl2 (25 mM), and primers (10 μM) were 0.5, 1.2, and 2 μl, respectively, for 25 μl reaction mixtures. Bovine serum albumin was also added (final concentration, 0.2 μg/ml). The touchdown PCR (31 cycles) was from Promega, which also provided the 10× buffer. Thermal cycling conditions typically consisted of a touchdown series in which the annealing temperature decreased from 65 to 55°C by 1°C per cycle, followed by 15 cycles at 55°C, each for 1 min. The denaturing step was 1 min at 95°C, and the extension was 2.5 min at 72°C. The PCR products were concentrated by centrifugation with Microcon filters (Amicon) and cloned with a TOPO-TA cloning kit with pcRII-TOPO (Invitrogen) according to the manufacturer’s instructions.

### Screening the general bacterial and CF libraries.

All white (insert-bearing) colonies were picked from the general bacterial library and placed into 96-well microtiter plates. The insert length in 10 random clones was examined by PCR as described below, and representative clones were sequenced.

#### Screening the Arctic Ocean.

The 16S rRNA genes from representatives of each RFLP type were compared with the Cytophaga-Flavobacterium sequences in the 16S rRNA gene database (pgene42a ga31 3.0). Target Reference:

<table>
<thead>
<tr>
<th>Oligonucleotide</th>
<th>Sequence (5’→3’)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>EubA</td>
<td>AAGAGGTTGATCCACNCRCRA</td>
<td>Bacteria</td>
</tr>
<tr>
<td>EubB</td>
<td>AGATTTGATCMGTGCGTAGC</td>
<td>Bacteria</td>
</tr>
<tr>
<td>EubA338</td>
<td>GCITGCCTCCGATTAGGAGT</td>
<td>Bacteria</td>
</tr>
<tr>
<td>Ald968</td>
<td>GGTAAAGGTTTCGGCGGTT</td>
<td>Alpha-proteobacteria</td>
</tr>
<tr>
<td>Beta42a</td>
<td>GCCTTCCCCACCTGTITTT</td>
<td>Beta-proteobacteria</td>
</tr>
<tr>
<td>Gama42a</td>
<td>GCCTTCCCCACCTGTITTT</td>
<td>Gamma-proteobacteria</td>
</tr>
<tr>
<td>CF319a</td>
<td>TGTTCCGCTGTCTAGTAC</td>
<td>Universal CF</td>
</tr>
<tr>
<td>CF356</td>
<td>GGACCTTAAAACCCCAT</td>
<td>Universal CF</td>
</tr>
<tr>
<td>CF316</td>
<td>CTGGTACGTGAACACOGA</td>
<td>Universal CF</td>
</tr>
<tr>
<td>CF6-1267</td>
<td>GAAGATCTGCTCCTCCTC</td>
<td>CF subgroup (DE cluster 2)</td>
</tr>
</tbody>
</table>

a Universal CF, all members of the Cytophaga-Flavobacterium group.
pleated sequenced. Sequences from this study (approximately 1,150 bases) and from a previous study of Cytophaga-like bacteria (33) (approximately 1,000 bases) were added to the rRNA sequence database of the Technical University of Munich (version 6, spring 2001) by using the ARB package (http://www.mikro.biologie.tum.de/arb). A neighbor-joining tree of this database consisting of the new sequences, Bacteroidetes sequences in the ARB database, and Chlorobium limicola (the outgroup) were aligned with the ARB FastAligner version 1.03, adjusted manually with secondary-structure criteria, and then analyzed phylogenetically with the neighbor-joining algorithm. Highly variable positions in the aligned sequences were removed by using the E. coli filter in ARB. Confidence in the phylogeny topology was inferred from an analysis of 100 bootstrap replicates.

The taxonomic distributions of the RFLP types in the two libraries were compared with a R × C likelihood ratio test by the use of a Monte Carlo method to obtain an unbiased estimate of the exact P value, based on a Monte Carlo sample of 10,000 tables from the reference set. The software used for this statistical test was StatXact 4 for Windows (CYTEL, Cambridge, Mass.).

### Design and application of oligonucleotides for FISH detection of Cytophaga-like bacteria

One clade of Cytophaga-like bacteria was represented by several clones in both the Cytophaga-like library and the general bacterial library (see Results). The ARB probe tool was used to design oligonucleotide probes to detect this clade. The hybridization conditions were optimized in dot blot assays and were used in FISH assays with cultured bacteria. Tests with cultured Cytophaga-like bacteria not belonging to this clade indicated that nonspecific hybridization was minimized with a 20% formamide concentration. The NaCl concentration of the wash solution was 308 mM.

Samples for FISH analyses were taken along the salinity gradient of the Delaware Estuary in March 2001 and in a depth profile of the Chukchi Sea (73°56.6′N, 160°33′0′′W) in May 2002. Water was preserved in 2% fresh paraformaldehyde for about 24 h, after which they were filtered through polycarbonate filters, which were then frozen until analysis. Hybridization and wash conditions for FISH analyses with CY3 (MOW Biotech, Inc.)-labeled probes were as described previously (9). The probes for the alpha-, beta-, and gamma-proteobacteria were Al968, Beta42a, and Gama42a, respectively; CF319a was used for Cytophaga-like organisms (Table 1). A negative control probe was also routinely used. To estimate the abundance of bacteria that were not in these four groups, the relative abundances in the four groups were summed and then subtracted from the abundance determined by the general bacterial probe (Eub338). The standard error of the estimate for other bacterial groups was calculated with a propagation-of-error analysis (7), applied to the counting errors for the four groups and for the Eub338-positive cells.

Filters for the FISH analyses were analyzed with a semiautomated image analysis system coupled to a charge-coupled-device camera mounted on an Olympus Provis AX70 epifluorescence microscope (10), which counts DAPI (4′,6-diamidino-2-phenylindole)-positive cells and CY3-positive cells that correspond to DAPI-positive cells. Biovolumes were calculated by digital integration (39) of the DAPI-stained cells by using the image analysis system calibrated with 0.5-μm-diameter yellow fluorescent beads (Polysciences). Cell sizes reported for prokaryotes were measured on all DAPI-stained cells in FISH preparations. The number of cells measured varied from about 100 (for the Cytophaga-like subgroup) to >1,000 (for total prokaryotes).

### Nucleotide sequence accession numbers

The sequences of all 16S rRNA genes have been deposited in GenBank under the accession numbersAY274835 to AY274871, organized here in groups defined by the ARB analysis: DE cluster 2 with clones CF4, CF6, CF91, CF20, SB26, SB31, and SB45 (accession no. AY274856, AY274860, AY274861, AY274860, AY274860, AY274870, and AY274871, respectively); Microbiella cluster with clones CFI7, CF44, CF80, CF92, CF69, CF101, CF108, IA5, and IG12 (AY274849, AY274855, AY274866, AY274865, AY274866, AY274865, AY274866, and AY274870, respectively); Polaribacter cluster with clones CF10, CF18, and CF57 (AY274847, AY274865, and AY274857, respectively); DE cluster 1 with clones CF30, CF58, CF77, 2C, and 2D9 (AY274852, AY274855, AY274864, and AY274841, respectively); Cellulophaga cluster with clones CF2 and 2D10 (AY274851 and AY274838, respectively); Flavobacterium cluster with clone CF39 (AY274854); and two unclassified clones, CF100 (AY274844) and CF36 (AY274853).

### RESULTS

**Design of primers for Cytophaga-like bacteria.** We hypothesized that PCR primers targeting Cytophaga-like bacteria retrieved more novel 16S rRNA genes from natural bacterial assemblages than general bacterial primers. To examine this hypothesis, we first tried the complement to the common FISH probe (CF319a-F) paired with a general bacterial primer (EUBa) (Table 1) in PCR. Unfortunately, this primer set amplified 16S rRNA genes from bacteria outside of the Bacteroidetes division (data not shown). To minimize this nonspecificity, we designed a new primer (CF316) which differs from CF319a-F only in the positions of the mismatches; mismatches at the 3′ end of the primer help to prevent extension and thus amplification of nonspecific products. The MgCl₂ concentration was decreased to 1.2 mM to further reduce nonspecific amplification. When used in PCR, this new primer (CF316), paired with EUBa, amplified 16S rRNA genes only from isolates of Cytophaga-like bacteria (four were tested), not proteobacteria; we tested three isolates each from the alpha, beta, and gamma subdivisions of proteobacteria. These primers were then used in a PCR with DNA isolated from the Delaware Estuary. Nearly all of the 110 clones in the resulting CF library carried 16S rRNA genes from the Bacteroidetes division, but three did not. These results indicate that the new primer pair targeting Cytophaga-like bacteria resulted in a library greatly enriched in Cytophaga-like 16S rRNA genes, but these genes must be sequenced to ensure that they came from bacteria that indeed belong in the Bacteroidetes division.

### Characterization of the CF and general bacterial libraries

Two libraries of 16S rRNA genes were constructed using DNA isolated from the bacterial size fraction collected from the Delaware Estuary. The general bacterial library was dominated by alpha-proteobacteria (>60% of all clones), as is typical for marine waters, including the Delaware Estuary (9), although this bacterial group comprised only about 20% of total prokaryotic abundance as determined by FISH (Fig. 1). Cytophaga-like bacteria also comprised about 20% of total prokaryotic abundance as determined by FISH, but they made up only about 5% of the clones in the general library (Fig. 1). Compared to the alpha-proteobacteria and the Cytophaga-like bacteria, the gamma- and beta-proteobacteria were less abundant among the FISH-detectable bacteria and in the general bacterial library (Fig. 1).

The second library consisted of amplicons from a PCR with CF316 and EUBa, which was designed to retrieve 16S rRNA genes from the Cytophaga-Flavobacterium group (CF library). This library consisted of 107 clones of Cytophaga-like 16S rRNA genes which could be divided into 21 groups based on RFLP analysis (Table 2). In contrast, of the 228 clones in the general bacterial library, probing and sequencing indicated that only 11 were from Cytophaga-like bacteria, which were represented by six RFLP types (Table 2). Representatives of each RFLP type from both libraries were fully sequenced and analyzed with ARB (Fig. 2).

The Cytophaga-like clones were classified into six clades, except for two clones from the CF library (CF100 and CF36) that could not be put into any group (Table 2). The two libraries did not differ significantly in how the RFLP types were distributed among the seven groups (P > 0.05; R × C likelihood ratio test). The Cytophaga-directed primer pair retrieved more Cytophaga-like 16S rRNA genes than were found in the general bacterial library, but these genes did not qualitatively differ from those retrieved with general bacterial primers; there was no statistical difference in the taxonomic distribu-

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CF6 (accession no. AY274860) is the sequenced representative with the most abundant RFLP type having 17 clones; clone the CF library by 28 clones divided among four RFLP types, most clones (Table 2). The entire cluster was represented in the CF library and belonged to DE cluster 2, which also had the most clones in the Delaware Bay (Fig. 2). The most common RFLP type was defined initially only by sequences of the Cytophaga-like 16S rRNA genes retrieved by the two PCR primer sets.

A large fraction (38%) of all Cytophaga-like clones from our two libraries could be grouped into two clusters (DE clusters 1 and 2) (Table 2) that were defined initially only by sequences from the Delaware Bay (Fig. 2). The most common RFLP type in the CF library belonged to DE cluster 2, which also had the most clones (Table 2). The entire cluster was represented in the CF library by 28 clones divided among four RFLP types, with the most abundant RFLP type having 17 clones; clone CF6 (accession no. AY274860) is the sequenced representative of this RFLP type. DE cluster 2 was represented by only one clone in the general library. The second-most-common RFLP type in this library (13 clones) was classified in the Cellulophaga cluster, which contain bacteria from the Delaware Estuary as well as other environments. The Microscilla and Polaribacter clusters had more clones than the Cellulophaga cluster, but clones in these two clusters were distributed among more RFLP types (seven and three, respectively) than in the Cellulophaga cluster, which had only one RFLP type in the CF library (Table 2). The most common RFLP type in the general library was found in DE cluster 1 and was represented by six clones (Table 2).

As expected, the diversity of RFLP types in the CF library was lower than is typically observed in general bacterial libraries, according to three of four common diversity indices (Table 3); the evenness index was higher for the CF library than for the other libraries. Of more interest, the rarefaction curve for the CF library leveled off and approached a maximum after about 80 clones, whereas the curve for the general library did not (Fig. 3). Likewise, the coverage of the CF library was 91%, which is substantially higher than the coverage for general libraries from seawater or soils (Table 3). These data suggest that the size (number of clones) of the CF library was large enough to adequately sample these bacteria but that the general bacterial library of about 100 clones did not adequately sample the entire diversity of 16S rRNA genes in this sample from the Delaware Estuary.

Abundance of Cytophaga-like bacteria in the Delaware Estuary and Arctic Ocean. Representatives of DE cluster 2, which was the most abundant clone type in the CF library (Table 2), were sequenced, and a FISH oligonucleotide probe (CF6-1267) (Table 1) was designed to match with no mismatches to three (CF6, CF20, and CF91) of the eight sequences available in this cluster. There are two to three mismatches between the probe and the other DE cluster 2 sequences.

The FISH analysis confirmed that DE cluster 2 was present in the Delaware Estuary in March 2001 (Fig. 4) and in the Arctic Ocean (Fig. 5). Bacteria from DE cluster 2 comprised about 5% of the total prokaryotic abundance and varied from undetectable (compared to the detection level in a negative

![FIG. 1. Composition of a general 16S rRNA gene library (percent-](Image 67x511 to 277x722)

### TABLE 2. Taxonomic affiliations of Cytophaga-like clones in the general bacterial and selected Cytophaga-like libraries constructed with DNA from the Delaware Bay

<table>
<thead>
<tr>
<th>Taxonomic group</th>
<th>No. of clones (no. of RFLP types)</th>
<th>Sequenced clone name(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CF library</td>
<td>General library</td>
</tr>
<tr>
<td>DE cluster 2</td>
<td>28 (4)</td>
<td>1 (1)</td>
</tr>
<tr>
<td>Microscilla cluster</td>
<td>20 (7)</td>
<td>2 (2)</td>
</tr>
<tr>
<td>Polaribacter cluster</td>
<td>14 (3)</td>
<td>0</td>
</tr>
<tr>
<td>DE cluster 1</td>
<td>13 (3)</td>
<td>6 (2)</td>
</tr>
<tr>
<td>Cellulophaga cluster</td>
<td>13 (1)</td>
<td>2 (1)</td>
</tr>
<tr>
<td>Flavobacterium cluster</td>
<td>12 (1)</td>
<td>0</td>
</tr>
<tr>
<td>Unclassified (CF100)</td>
<td>5 (1)</td>
<td>0</td>
</tr>
<tr>
<td>Unclassified (CF36)</td>
<td>2 (1)</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>107 (21)</td>
<td>11 (6)</td>
</tr>
</tbody>
</table>

* The total numbers of clones and of clone types (RFLP types, shown in parentheses) in each taxonomic group are given. The groups were defined by analysis with ARB, as presented in Fig. 2.

* Names of clones bearing 16S rRNA genes sequenced during this study. DE cluster 2 also includes sequences from our Arctic library (SBI5, SBI26, and SBI31) and another Arctic library (97A-14) from a previous study (6). The accession numbers are provided in the text.
control) to 10% of the total abundance in the estuary (Fig. 4). The contribution of this subgroup to the total number of Cytophaga-like bacteria detected by the CF319a probe varied from insignificant to as much as a 40% representation, with an overall average of 21% (Fig. 4). The bacteria detected by the CF6-1267 probe varied much more than the total Cytophaga-like bacterial abundance; the coefficient of variation for the subgroup of Cytophaga-like bacteria was over twice that of the total group (>70% versus 25%).

This Cytophaga-like bacterial subgroup was also present in
the Arctic Ocean (Fig. 5). The number of total Cytophaga-like bacteria (CF319a) did not vary substantially with depth and comprised 21 to 29% of total prokaryotic abundance. DE cluster 2, identified by the CF6-1267 probe, also did not vary systematically with depth and comprised about 15% of the total abundance. This Cytophaga-like bacterial subgroup comprised about 50% of the total Cytophaga-like bacteria (CF319a probe) throughout the upper 60 m at this Arctic station. To confirm that DE cluster 2 was present in the Arctic Ocean, the Cytophaga-Flavobacterium-targeted primers (CF316 and EubA) were used in a PCR with an Arctic surface water sample, and the amplicons were cloned. We found that the second-most-abundant group of bacterial 16S rRNA genes in the Arctic CF library was most similar to DE cluster 2 (Fig. 2). The Arctic 16S rRNA genes (representing three RFLP types) were 94.9 to 96.4% similar to other 16S rRNA genes in DE cluster 2, about the same overall similarity as within this cluster (94.7 to 99.8%). The DE cluster 2 accounted for 9% of the 85 clones screened in the Arctic CF library. These results confirm the results of the FISH analysis indicating the presence of DE cluster 2 in the Arctic Ocean.

Bacteria in DE cluster 2 of Cytophaga-like bacteria were larger than other microbes in both the Delaware Estuary and the Arctic Ocean. In the Delaware Estuary, the DE cluster 2 bacteria (CF6-1267-positive cells) were about 0.05 μm³, which was 1.6-fold larger than the average bacterium or prokaryote (Table 4). The average cell volumes for the total Cytophaga-like bacterial assemblage (CF319a-positive cells) and DE cluster 2 (CF6-1267-positive cells) were significantly larger than those of the entire bacterial assemblage (Eub338-positive cells) and of the entire prokaryotic community (DAPI-positive cells) (P < 0.001; t test). The cell volumes for the total Cytophaga-like bacterial assemblage and DE cluster 2 did not differ significantly in the Delaware Estuary (P > 0.05). In the Arctic Ocean, the CF319a-positive bacteria were significantly smaller than the average bacterium and prokaryote (P < 0.001; t test) (Fig. 6), but as seen in the Delaware Estuary, the DE cluster 2 bacteria were large (about 0.06 μm³). These bacteria were significantly larger than the CF319a-positive bacteria, and they were also larger than the average bacterium (Eub338-positive cells) and prokaryote (P < 0.001; t test), although the difference was only about 1.4-fold (Fig. 6). These data indicate that

![FIG. 3. Rarefaction curves for the general and CF libraries from the Delaware Estuary.](image)

![FIG. 4. Relative abundances of all bacteria (Eub338-positive cells), Cytophaga-like bacteria (CF319a-positive cells), and DE cluster 2 bacteria (CF6-1267-positive cells) in the Delaware Estuary in March 2001. The data are expressed as percentages of the total number of DAPI-positive cells (prokaryotes). The dashed horizontal line is the average negative control. The error bar indicates the standard error. Distance Upstream, the distance from Cape Henlopen, which is at the mouth of the estuary.](image)
the contribution of DE cluster 2 bacteria to total bacterial biomass is larger than is indicated by their abundance.

**DISCUSSION**

The biogeography of the alpha- and beta-proteobacteria in aquatic ecosystems is becoming clear (21), in part because both groups are well represented in clone libraries of 16S rRNA genes from marine and freshwater habitats (20) and also because several oligonucleotides suitable for FISH- and PCR-based methods are available for detecting these bacteria (3, 32). Because of this previous work, much is known about a few clades within the alpha-proteobacteria, such as *Roseobacter* spp. (22) and SAR11 (32, 37). Analogous data on *Cytophaga*-like bacteria are sparse, although it is known that the abundance of the entire group can rival that of alpha-proteobacteria in aquatic systems (21, 27). Nearly all estimates of the abundance of uncultured *Cytophaga*-like bacteria come from FISH assays using a single oligonucleotide probe (CF319a) (21, 27). Since this probe does not recognize all *Cytophaga*-like bacteria (33, 44), alternatives have been suggested (33, 44), but there

**TABLE 4. Biovolumes of all prokaryotes, bacteria, and *Cytophaga*-like bacteria in the Delaware Estuary in March 2001**

<table>
<thead>
<tr>
<th>Distance(^b) (km)</th>
<th>Prokaryotes</th>
<th>Bacteria</th>
<th><em>Cytophaga</em>-like bacteria</th>
<th>DE cluster 2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DAPI</td>
<td>Eub338</td>
<td>CF319a</td>
<td>CF6-1267</td>
</tr>
<tr>
<td>9</td>
<td>0.0337</td>
<td>0.0387</td>
<td>0.0455</td>
<td>0.0530</td>
</tr>
<tr>
<td>29</td>
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<td>0.0354</td>
<td>0.0611</td>
<td>0.0650</td>
</tr>
<tr>
<td>44</td>
<td>0.0341</td>
<td>0.0364</td>
<td>0.0578</td>
<td>0.0714</td>
</tr>
<tr>
<td>66</td>
<td>0.0319</td>
<td>0.0314</td>
<td>0.0537</td>
<td>0.0413</td>
</tr>
<tr>
<td>82</td>
<td>0.0262</td>
<td>0.0306</td>
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<td>100</td>
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</tr>
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<td>0.0252</td>
<td>0.0393</td>
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<td>0.0308</td>
<td>0.0448</td>
<td>0.0521</td>
</tr>
<tr>
<td>142</td>
<td>0.0337</td>
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\(^a\) Values are mean cell volumes (± standard errors) detected with the indicated stain or probes. Eub338, CF319a, and CF6-1267 are FISH probes for all bacteria, all *Cytophaga*-like bacteria, and DE cluster 2 (a subgroup of *Cytophaga*-like bacteria), respectively. DAPI detects all prokaryotes.

\(^b\) Samples were taken at the indicated distance from the mouth of the estuary (distance equal to zero). Over the entire estuary, the DE cluster 2 bacteria were significantly larger than the average size of all prokaryotes and bacteria (\(P < 0.05\)).
has been little work on subgroups within the *Cytophaga-Flavobacterium* group in aquatic systems (16).

Results from the clone libraries constructed during this study should help identify some of these subgroups, which may be abundant in natural environments like estuaries and the oceans. The CF library, which was constructed with amplicons from a PCR targeting the *Cytophaga-Flavobacterium* group, had nearly 10-fold more 16S rRNA genes from this group than the general bacterial library. Most of these 16S rRNA genes could be classified with subgroups defined by bacteria or uncultured representatives from other environments; they were not unique to the Delaware Estuary. However, the cluster with the largest number of clones in the CF library (DE cluster 2) initially contained sequences from only the Delaware Estuary, although we subsequently found it in an Arctic CF library as well. This cluster alone accounted for about 26% (28 of 107) of all sequences in the Delaware CF library; a similar fraction of all *Cytophaga*-like bacteria (CF319a) was detected by the FISH probe targeting DE cluster 2 (21% ± 13%, average for the entire estuary). A recently discovered freshwater subgroup (TAF) of the *Cytophaga-Flavobacterium* cluster (33) was not observed in our libraries of marine bacterial 16S rRNA genes.

In addition to being from freshwater, the TAF sequences were found in a library constructed from an epilithon community (33), whereas our libraries are from pelagic communities.

DE cluster 2 organisms were detected in the Chukchi Sea (Arctic Ocean) by FISH analysis and in a *Cytophaga-Flavobacterium*-targeted clone library of 16S rRNA genes. These bacteria were also detected by a recently published study of bacterial diversity in the Arctic Ocean (6). Bano and Hollibaugh (6) found eight *Cytophaga*-like 16S rRNA genes in general bacterial libraries of Arctic surface waters; our analysis indicates that one of these eight belongs in DE cluster 2 (Fig. 2). Other studies have shown that the *Cytophaga-Flavobacterium* group is abundant in polar waters, according to both FISH analysis (40, 45, and this study) and culture-based work (26). While the Delaware Estuary and the Arctic Ocean differ in many respects, the Chukchi Sea has some estuarine properties, including the impacts of rivers (1), close coupling between the water column and the benthos (24), and high primary production (8). The Delaware Estuary was sampled in March when the water temperature was about 6°C, which is substantially warmer than the Chukchi Sea (about 0°C) but perhaps cold enough to select for psychrophilic *Cytophaga*-like bacteria.

We did not use the general FISH probe (CF560) for the *Cytophaga-Flavobacterium* cluster as suggested by O'Sullivan et al. (33), but it probably would have recognized many bacteria in our samples, with some important exceptions. The CF560 probe probably would not recognize bacteria represented by four of our clones (3G9, CF16, CF61, and CF4) because there are three to five mismatches between the 16S rRNA genes of these clones and the CF560 probe. The four mismatches with CF4 are particularly noteworthy because this clone is in DE cluster 2, the most abundant subgroup of *Cytophaga*-like bacteria in the Delaware Estuary. These data illustrate the difficulty in attempting to devise probes that will recognize all environmentally relevant *Cytophaga*-like bacteria.

In spite of this study and previous work (33, 44), the phylogeny of the *Cytophaga-Flavobacterium* cluster is still difficult to access, as is evident from the low bootstrap values in phylogenetic trees (e.g., Fig. 2). Perhaps because of problems in constructing the phylogeny of this cluster, we were also unsuccessful in designing specific oligonucleotide FISH probes for another *Cytophaga*-like subgroup, the *Cellulophaga* subgroup. More sequence information would help in designing probes for this and other *Cytophaga*-like subgroups. Although not tested in the FISH assays here, the *Cytophaga*-like probe used by Eilers et al. (16) probably would have recognized bacteria at least in DE clusters 1 and 2 and the *Cellulophaga* cluster; their CYT1448 probe binds perfectly to our 16S rRNA sequences in these clusters.

In spite of the limitations of the data sets currently available for *Cytophaga*-like bacteria, we were able to identify a couple of subgroups of these bacteria that appear to be abundant in marine surface waters. In particular, FISH data indicate that bacteria in DE cluster 2 account for about 10% of the total prokaryotic abundance in the Delaware Estuary and Arctic Ocean. While apparently less abundant than SAR11 (18% of 16S rRNA genes, 35% of cells detected by FISH analysis in a depth profile [32]), DE cluster 2 bacteria are still a substantial fraction of the community, and the abundance of this subgroup is equal to or even higher than that of other bacteria at this phylogenetic level, for example, SAR86 and other gamma-proteobacteria (9, 15). The subgroup of *Cytophaga*-like bacteria detected by the CYT1448 probe (16) was about 5% of the total number of cells in the North Sea (16), although that probe may bind to a couple of diverse subgroups (see above). In contrast, the abundance estimate for DE cluster 2 bacteria is conservative because the CF6-1267 probe for these bacteria matches only three of the eight sequences available in the cluster. Use of multiple probes, analogous to the four FISH probes used for quantifying SAR11 (32), may lead to higher abundance estimates for DE cluster 2.

The contribution of DE cluster 2 *Cytophaga*-like bacteria to total prokaryotic biomass is probably larger than is indicated by the abundance estimates alone. The average cell volume of DE cluster 2 bacteria was larger than that for other bacteria and prokaryotes in this study even when the average cell volume of all *Cytophaga*-like bacteria was not particularly large. DE cluster 2 bacteria (0.05 to 0.06 μm³) also appear to be substantially larger than bacteria in the SAR11 clade, which have been reported to be about 0.01 μm³ (37). These estimates may not be directly comparable because, in addition to the two studies using samples from different seasons and habitats, our cell volumes were estimated with DAPI-fluorescing cells, whereas the SAR11 estimate is from electron micrographs (37), although even the average size of DAPI-fluorescing SAR11 cells apparently is smaller than that of other bacteria (32). Both approaches may underestimate cell biovolumes because DAPI does not stain the entire cell (36, 42) and because of shrinkage during the preparation of samples for electron microscopy (17). However, even if the absolute cell size is incorrect, it is difficult to see how methodological problems with measuring DAPI-stained cells could lead to an erroneous conclusion about DE cluster 2 bacteria being bigger than the average bacterium.

Assuming that this difference in cell size is confirmed by more data, such a finding suggests differences in the regulation of growth and mortality of DE cluster 2 bacteria versus other bacteria, such as those in the SAR11 clade. A small size would
be an advantage in oligotrophic environments like the open oceans, where labile DOM concentrations are low (37). In contrast, DE cluster 2 bacteria may be large because they use high-molecular-weight DOM, which occurs in relatively high concentrations in aquatic habitats (4, 5). Whether high-molecular-weight DOM is used by DE cluster 2 bacteria remains unknown, but other Cytophaga-like bacteria are well known for their capacity to use biopolymers and other large DOM molecules (11, 27, 38). Bacteroides thetaiotaomicron, which is in the same division as Cytophaga-like bacteria, has a large number of genes encoding enzymes involved in polysaccharide hydrolysis and mineralization (46), but cell size and abundance may not necessarily reflect only bottom-up factors, such as the supply of DOM. Grazing and viral lysis are known to select against large microbial cells (23, 34, 43). M. T. Cottrell and D. L. Kirchman argued that grazing was the most likely mechanism to explain their observation of a negative relationship between the average cell size of a bacterial group and its relative abundance in the Delaware Estuary (10a). Regardless of the actual mechanism, the cell size data suggest that DE cluster 2 bacteria are regulated differently than other bacteria in the Delaware Estuary and Arctic Ocean.

More work is obviously needed to assess the abundance of various bacterial groups in aquatic environments and to examine their role in DOM uptake and other ecological processes. We have some information about the abundance of broad phylogenetic groups (the subdivisions of Proteobacteria and Cytophaga-Flavobacterium organisms), but have only limited data on finer phylogenetic levels, such as those of SAR11 and the clusters of Cytophaga-like bacteria examined here. Although much attention has been paid to SAR11 and another alpha-proteobacterial clade (Roseobacter), other subgroups of this proteobacterial subdivision are found in clone libraries from bacterial communities in surface marine waters (20). Likewise, DE cluster 2 appears to be one of several subgroups of the Cytophaga-Flavobacterium group. This cluster accounted for about 25% of all Cytophaga-like bacterial clones, which is about the fraction accounted for by Cytophaga-like bacteria according to FISH. The second-most-abundant clone family (the Microscilla cluster) accounted for another 18% of the clones in the CF library, leaving the remaining 57% spread out over six clusters. Determining the relative abundance of these bacterial groups is important for examining their role in DOM uptake and other ecological processes.

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REFERENCES


