Biogeography of major bacterial groups in the Delaware Estuary

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Abstract

We used fluorescence in situ hybridization to examine the spatial and temporal variation in the abundance of major bacterial groups in the Delaware Estuary. The abundance of alpha- and beta-proteobacteria and *Actinobacteria* varied systematically in the estuary and mirrored the pattern seen in lakes and oceans. Beta-proteobacteria and *Actinobacteria* were abundant in the Delaware River but were less so in the marine waters of the Delaware Bay. In contrast, alpha-proteobacteria, including the SAR11 clade, were most abundant in the Bay and rare in the Delaware River. *Actinobacteria* were active in assimilating thymidine and leucine and appeared to contribute substantially to bacterial production in the Delaware River. Among the several biogeochemical parameters we examined, only salinity accounted for a substantial portion of the variation in abundance of these bacterial groups. However, relative abundance of these groups often varied independently of salinity. *Cytophaga*-like bacteria were often abundant throughout the estuary, but they did not vary systematically over the estuarine gradient, unlike the other dominant bacterial groups. We hypothesize that this estuary-wide high abundance occurs because *Cytophaga*-like bacteria are very diverse, more so than other groups. Data on 16S rRNA sequences are consistent with this hypothesis. The consistent biogeographic patterns suggest that some bacterial groups, even at a broad phylogenetic level, operate as ecologically meaningful units for examining some processes, whereas the *Cytophaga*-like bacteria as now defined might be too diverse to be useful for ecological studies.

The biogeography of major bacterial groups in aquatic ecosystems is now being revealed through the use of various culture-independent methods. We probably know the most about the biogeography of two proteobacterial groups. Alpha-proteobacteria are often the most abundant group in the euphotic zone of the oceans but are much less common in freshwater lakes, whereas the opposite is the case for beta-proteobacteria (Glöckner et al. 1999). The high-GC gram-positive group, *Actinobacteria,* is also abundant in lakes (Glöckner et al. 2000), perhaps more so than in the oceans (Acinas et al. 2004; Venter et al. 2004). A subgroup of bacteria in the Bacteroidetes division, often called *Cytophaga-Flavobacter,* but here referred to as *Cytophaga*-like bacteria, appears to be abundant in both lakes and the oceans (Glöckner et al. 1999; Kirchman 2002). It is unclear why there are consistent biogeographical patterns for some bacterial groups and not for others.

The spatial distribution of alpha- and beta-proteobacteria in estuaries is similar to that seen in lakes and the oceans. Alpha-proteobacteria dominate the marine end of estuaries, but their abundance is low in low-salinity waters, whereas the pattern is the reverse for beta-proteobacteria (Bouvier and Del Giorgio 2002; Cottrell and Kirchman 2003). *Cytophaga*-like bacteria have been found associated with the turbidity maximum of the Columbia River (Crump et al. 1999) and in two subestuaries of the Chesapeake Bay (Bouvier and Del Giorgio 2002), but otherwise these bacteria do not appear to vary systematically in estuaries. The abundance of *Actinobacteria* has not been examined quantitatively in estuaries to date, although their 16S rRNA genes have been detected (Crump et al. 2004).

These consistent biogeographical patterns at such a high phylogenetic level are surprising. Each of the bacterial groups mentioned above potentially consists of hundreds to thousands of different bacteria as defined by 16S rRNA sequences (Giovannoni and Rappé 2000). Even more types of bacteria are distinguishable with the use of other genes that resolve at a finer phylogenetic level (Santos and Ochman 2004). One might expect that this diversity would ensure that some members in each group would be capable of flourishing in different aquatic environments. If so, then there would be no consistent biogeographical patterns among the proteobacterial subdivisions and *Cytophaga*-like bacteria. However, it is not clear whether these bacterial groups are equally diverse globally or in a particular environment or if phylogenetic diversity, as defined by 16S rRNA, is accompanied by phenotypic diversity. Estuaries offer an opportunity to begin to explore these questions.

The goals of this study were to examine the biogeography and seasonal variation of some major aquatic bacterial groups and select subgroups in the Delaware Estuary in order to provide basic information about these microbes and to explore mechanisms that lead to the observed biogeographical patterns at broad phylogenetic levels—that is, the division (or phylum) and subdivision level. Cottrell and Kirchman (2003) focused on thymidine and leucine incorporation and presented data on some bacterial groups at four stations within the Delaware River in March and June 2001. The data presented here are from 12 stations in March, June, August, and December 2001 and in March, June, and December 2002. We also examined *Actinobacteria* and SAR11, an abundant alpha-proteobacterial clade (Giovannoni and Rappé 2000). We found consistent patterns in the spatial distribution of alpha- and beta-proteobacteria and *Actinobacteria* in the Delaware Estuary but not in *Cytophaga*-like bacteria, which often were abundant throughout the estuary.

Table 1. Probes used to examine the abundance of major prokaryotic groups in the Delaware Estuary. A mixture of probes was used for SAR11 (Morris et al. 2002).

Phylogenetic group	Probe	Probe sequence	Reference
Archaea	Arch915	GTGCTCCCCCGCCAATTCCT	Amann et al. 1995
Bacteria	Eub338	GCTGCCTCCCGTAGGAGT	Amann et al. 1995
Alpha-proteobacteria	Alfa968	GGTAAGGTTCTGCGCGIT	Glöckner et al. 1999
Roseobacter	ROS537	CAACGCTAACCCCCT CC	Eilers et al. 2001
Beta-proteobacteria	Beta42	GCCTTCCCACTTCGTTT	Manz et al. 1992
Gamma-proteobacteria	Gam42a	GCCTTCCCACATCGTTT	Manz et al. 1992
Cytophaga-like	CF319	TGGTCCGTGTC CAGTAC	Manz et al. 1996
Actinobacteria	HGC69A	TATAGTTACCACCGCCGT	Glöckner et al. 2000
Actinobacteria	HGC840	TCGCASAAACCGTGGAAG	Glöckner et al. 2000
Negative control	NON	TAGTGACGCCGTCGA	Karner and Fuhrman 1997

Methods and Materials

Basic biogeochemical parameters—The Delaware Estuary was sampled in 2001 and 2002 as part of a multifaceted study of the biogeochemistry and microbial ecology of this estuary. Some of the basic environmental parameters were reported by Preen and Kirchman (2004) and were used here to explore relationships between bacterial community structure and biogeochemical processes. More details on the methods used for measuring the biogeochemical parameters can be found in Preen and Kirchman (2004). Primary production was estimated from $^{14}CO_2$ uptake over 24 h at five light levels obtained with the use of neutral-density screens and incubated at the in situ temperature in a deck incubator. Prokaryotic abundance was estimated by epifluorescence microscopy with 4′,6-diamidino-2-phenylindole (DAPI) staining. Incorporation rates of thymidine and leucine, which were used as indices of bacterial production, were estimated by the centrifugation method (Smith and Azam 1992).

Fluorescence in situ hybridization—Bacterial community structure was examined by fluorescence in situ hybridization (FISH) with oligonucleotide probes. Water for this analysis was preserved in fresh paraformaldehyde (2% final concentration) overnight and then filtered through 0.2-μm polycarbonate filters. The relative abundance of major phylogenetic groups was determined with CY3-labeled (MWG Biotech) probes (Table 1). The FISH protocol we used was described previously (Cottrell and Kirchman 2003) and included the use of unlabeled competitors when probing for beta- and gamma-proteobacteria. The abundance of SAR11 was determined via FISH with a suite of four probes (Morris et al. 2002) under the hybridization conditions described previously (Malmstrom et al. 2004). The FISH samples were analyzed with a semiautomated image analysis system coupled to an Olympus epifluorescence microscope (Cottrell and Kirchman 2003).

Microautoradiography combined with FISH—We used a combination of microautoradiography and FISH to examine the assimilation of leucine and thymidine by *Actinobacteria* in the Delaware River, where this microbial group is abundant (*see Results*). Samples for microautoradiography were taken from 1 m in the Delaware River ~200 km from the mouth of the estuary (the start of our transects) in June and December 2001. These samples were incubated with [^{3}H]thymidine and [^{3}H]leucine (20 nmol L^{-1}) at the in situ temperature for 4 h. Killed controls were poisoned with 2% paraformaldehyde. Incubations were terminated by adding 20% paraformaldehyde to a final concentration of 2% and filtered as described above for the FISH samples. The filters were then processed and analyzed as described by Cottrell and Kirchman (2003). The exposure time for microautoradiography was 8–14 h, which was the same as that used by Cottrell and Kirchman (2003).

Diversity of select bacterial groups—A clone library of 16S rRNA genes was constructed from the surface microbial community 10 km from the mouth of the estuary in March 2001. The water sample was first filtered through 1.0-μm polycarbonate filter (142 mm diameter) to remove eukaryotes and then this bacterial size fraction was collected onto Millipore Durapore filters (0.22 μm, type GVWP). The DNA was extracted by the standard phenol-chloroform extraction method, precipitated by sodium acetate and ice-cold isopropanol, and purified with a Geneclean kit (Qbiogene). The DNA was then used in a polymerase chain reaction with the general bacterial primers, EubA and EubB, and cloned as described previously (Cottrell and Kirchman 2000a). About 100 clones from this library were screened by sequencing a variable region of the 16S rRNA gene with the 907r primer. Ten clones bearing 16S rRNA genes from alpha-proteobacteria were randomly selected for complete sequencing.

PHYLIP was used to calculate the genetic distance (Jukes-Cantor method) (Felsenstein 1989) and the percent similarity for all possible pairings within each phylogenetic group. Then the grand average distance and percent similarity were calculated for each group. This analysis was also applied to previously collected sequence data from the Delaware River (Cottrell et al. in press) and to *Cytophaga*-like sequences from the Delaware Bay (Kirchman et al. 2003).

Statistical analysis—The data were analyzed by standard analysis of variance, regression, and correlation techniques. The FISH data were expressed as a percentage of total prokaryotes (DAPI-positive cells) and were arcsine transformed before analysis. Significance levels for correlations were

Table 2. Bacterial community structure as determined by FISH in the Delaware Estuary in 2001–2002. The statistics are for the entire data set and reflect both spatial and seasonal variation. The negative control was not subtracted from the other results.

Phylogenetic group	Probe	% of total prokaryotic abundance				
		Mean	SD	Mini-mum	Maxi-mum	n
Bacteria	Eub338	58	14	24	84	88
Alpha-proteobacteria	Alfa968	13	11	1	48	88
Beta-proteobacteria	Beta42	16	10	2	39	88
Gamma-proteobacteria	Gam42a	9	6	1	34	88
Cytophaga-like	CF319	16	10	2	44	83
Actinobacteria	HGC69A	13	10	1	41	51
Actinobacteria	HGC840	5	4	0	17	51
Negative control	NON	4	3	0	13	88
Total of groups*		74				

* Total refers to the sum of the alpha-, beta-, and gamma-proteobacteria, *Cytophaga*-like bacteria, and *Actinobacteria* (HGC69A results only).

modified for multiple comparisons with the Bonferroni adjustment.

Results

Environmental setting—We examined bacterial community structure along with several biogeochemical parameters likely to affect bacterial standing stocks and growth in the Delaware Estuary to gain insights into the factors controlling the relative abundance of major bacterial groups in estuarine and coastal ecosystems. Unlike the nearby Chesapeake Bay, freshwater flow into the Delaware Estuary is dominated by a single source, the Delaware River (51% of the total), and the entire estuary is usually well-mixed, except near the mouth of the estuary in summer (Sharp et al. 1982). The start of the salinity gradient, which is ~100 km upstream from the estuarine mouth, is also the start of a transition zone of high turbidity and of decreasing inorganic nutrient concentrations (Sharp et al. 1982; Preen and Kirchman 2004).

Community structure in the Delaware Estuary—The relative abundance of five major groups of bacteria in the estuary was determined by FISH, along with a positive control for all bacteria (Eub338 probe). Nearly 60% of all prokaryotes were recognized by the Eub338 probe on average (Table 2), but Archaea made up <10% in the estuary (9.4% ± 3.2%; $n = 12$). The Eub338-positive cells tended to be lower in the transition zone of the estuary (80–135 km; Fig. 1A), similar to what has been observed in the Chesapeake Bay system (Bouvier and Del Giorgio 2002). There were no significant correlations between Eub338-positive cells (percentage of total abundance) and temperature ($r = 0.07$, $p > 0.05$; $n = 87$) and between Eub338-positive cells and per cell rates of thymidine and leucine incorporation ($r = 0.16$ and $r = -0.05$, respectively, $p > 0.05$; $n = 74$).

The most abundant bacterial groups in the estuary were alpha- and beta-proteobacteria, *Cytophaga*-like bacteria, and *Actinobacteria*. Each of these groups accounted for ~15%
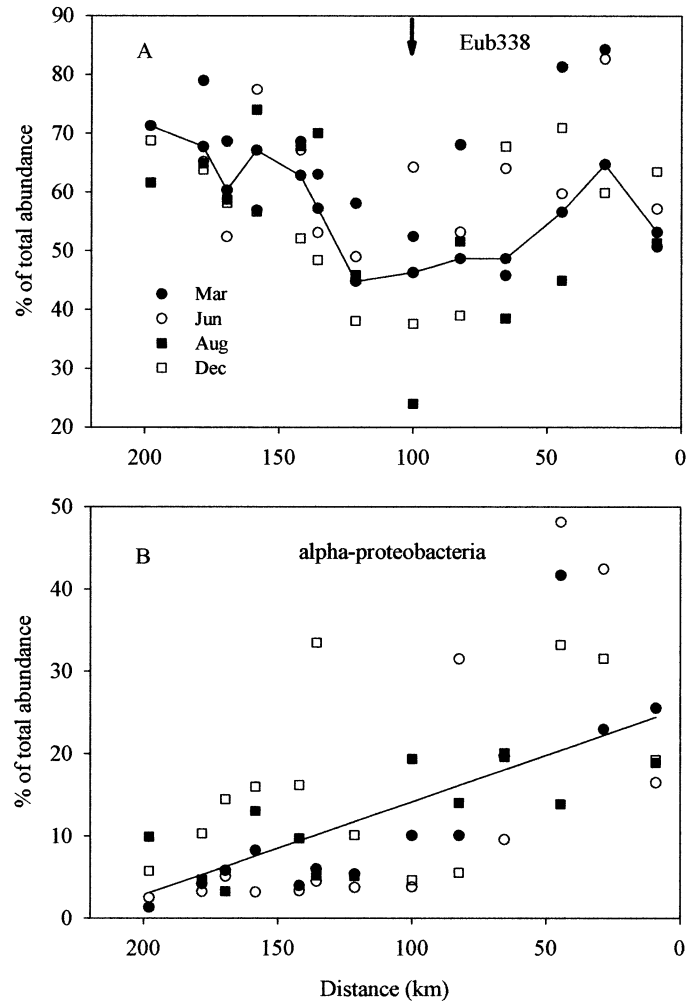

Fig. 1. Bacterial community composition in the Delaware Estuary in 2001. (A) Bacteria recognized by the general bacterial probe, Eub338; (B) alpha-proteobacteria. The line for Eub338 is the average for each location. The line for the alpha-proteobacteria is the regression line based on all of the data. The vertical arrow indicates the start of the salinity gradient and turbidity maximum. The initial point in the estuary (distance equal to zero) was defined as being the mouth of the estuary, between Cape May and Cape Henlopen.

of total prokaryotic abundance (Table 2), but there was much variation both spatially and temporally. Gamma-proteobacteria were nearly always the least abundant group that we examined and averaged ~9% of total prokaryotic abundance overall (Table 2). These five groups accounted for ~74% of prokaryotic abundance (the sum of the five averages; Table 2) and for roughly 100% of the Eub338-positive cells. To arrive at the latter conclusion, we subtracted the sum of the abundances for the five groups from the Eub338-positive cell abundance and calculated the average difference over the entire data set. The average difference was 8.8% ± 19%, indicating that usually the sum of the five groups was not significantly different from the relative abundance of the Eub338-positive cells.

The relative abundance of three groups (alpha- and beta-proteobacteria and *Actinobacteria*) varied substantially and
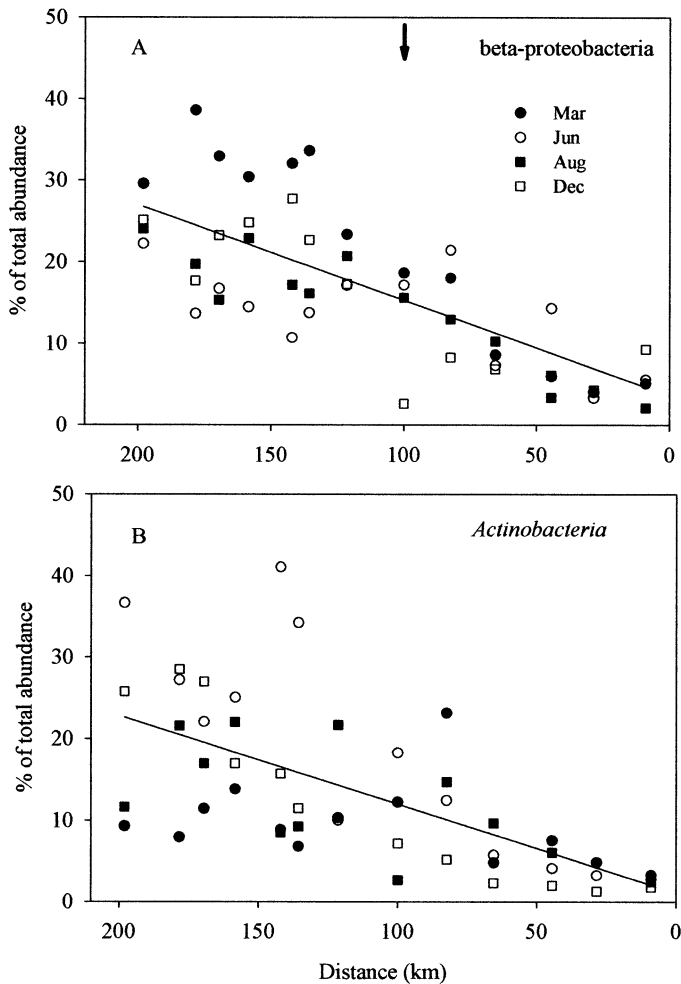
Fig. 2. Bacterial community composition in the Delaware Estuary in 2001. (A) Beta-proteobacteria; and (B) *Actinobacteria* recognized by the HGC69a probe. The lines for beta-proteobacteria and *Actinobacteria* are regression lines based on all of the data. The vertical arrow indicates the start of the salinity gradient and the zone of high turbidity.
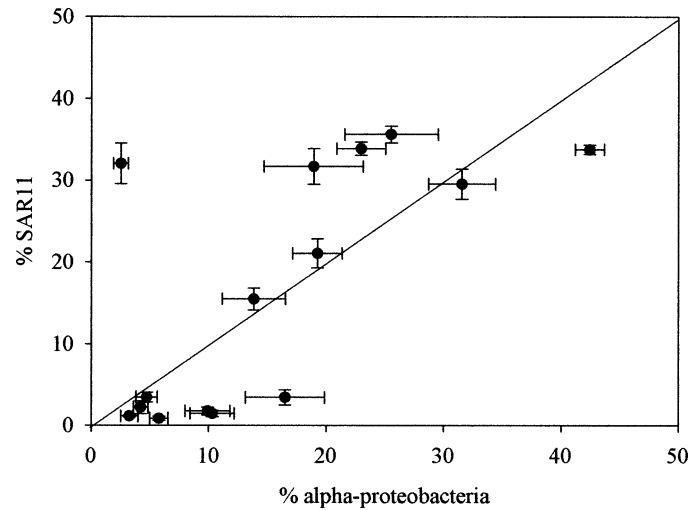

Fig. 3. Abundance of SAR11 and total alpha-proteobacteria as a percentage of total prokaryotic abundance. The line is the 1 : 1 line. The error bars are standard errors of 10 microscopic fields of view.

consistently along the estuarine gradient. The relative number of alpha-proteobacteria increased from <10% in the Delaware River to >20% near the mouth of the estuary (Fig. 1B). In the marine waters of the Delaware Bay, the abundance of this group exceeded 40% of total prokaryotic abundance in March and June. This percentage tended to be lower in the other months (Fig. 1B), but overall there was no consistent variation in alpha-proteobacterial numbers among the months we sampled. In contrast, beta-proteobacteria were most abundant in the freshwater region of the estuary and decreased in abundance along the estuarine gradient (Fig. 2A). Similar to alpha-proteobacteria, there was no obvious pattern to the variation in beta-proteobacterial abundance among the months we sampled (Fig. 2A).

The relative number of *Actinobacteria* detected by the HGC69a probe was also quite high in the Delaware River, ranging from 10% to over 40% of total prokaryotic abundance (Fig. 2B). The abundance of this group then decreased along the estuarine gradient and approached negative control

levels at the mouth of the estuary, similar to that of the beta-proteobacteria. Like the beta-proteobacteria, there was no significant difference in actinobacterial abundance among the seasons we sampled (Fig. 2B). The relative number of *Actinobacteria* detected by the HGC840 probe was much lower than that of bacteria detected by the HGC69a probe and was only slightly greater than negative control counts (Table 2). The abundance of the HGC840-positive bacteria correlated with the HGC69a-positive bacteria ($r = 0.62$, $n = 55$; $p \ll 0.001$).

The abundance of a prominent clade of alpha-proteobacteria, SAR11, was examined at two stations at the freshwater end and another two stations at the marine end of the estuary. SAR11 bacteria appeared to be abundant wherever alpha-proteobacteria were abundant (Fig. 3), and overall, SAR11 bacterial abundance was correlated with the abundance of alpha-proteobacteria ($r = 0.68$; $p < 0.005$; $n = 15$). Four points were above the 1 : 1 line in Fig. 4, suggesting problems with these probes, but only one was substantially away from the 1 : 1 line (>30% SAR11 and <5% alpha-proteobacteria). Excluding this point, SAR11 made up 77% of the alpha-proteobacterial abundance and >20% of total prokaryotic abundance in the marine waters, but <10% in the Delaware River. The clone library data from the marine end member also indicated that SAR11 made up a large fraction of the alpha-proteobacteria in the Delaware Estuary; of the 87 alpha-proteobacterial clones in this library, 78 clones (90%) could be placed in the SAR11 clade (*see Discussion*). *Roseobacter,* another abundant subgroup of alpha-proteobacteria, made up ~5% of total prokaryotic abundance in the estuary, according to results with the ROS537 probe ($5.4\% \pm 2.7\%$; $n = 12$).

The abundance of *Cytophaga*-like bacteria, which was one of the dominant groups in the estuary (Table 2), varied greatly both spatially and over time (Fig. 4). However, there was no clear trend over the estuarine gradient, except that many of the highest abundances were observed at the two marine
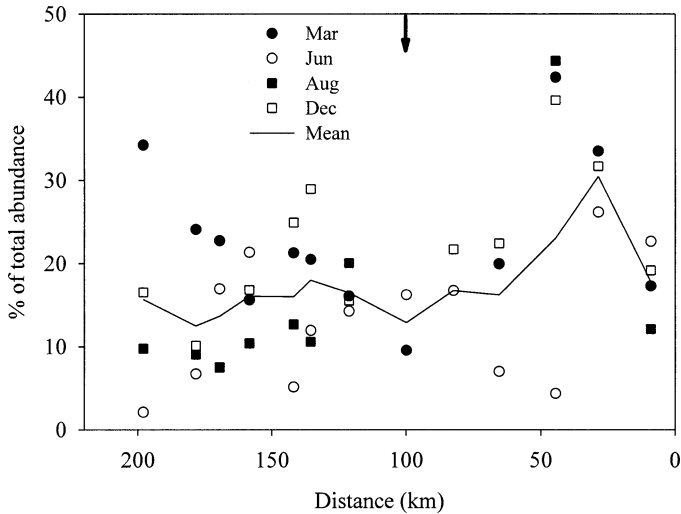
Fig. 4. Abundance of *Cytophaga*-like bacteria in the Delaware Estuary in 2001. The line indicates the mean for each station. The vertical arrow indicates the start of the salinity gradient and the zone of high turbidity.

stations (Fig. 4), and there was a small but significant correlation with salinity ($r = 0.35$, $p < 0.05$; $n = 82$). *Cytophaga*-like bacterial abundance varied significantly, as much as twofold, among the sample months ($p < 0.05$; ANOVA), but there was no clear pattern to that temporal variation. Likewise, the relative abundance of gamma-proteobacteria also varied significantly with time, but again not in an obvious pattern (data not shown).

Explaining the spatial patterns in bacterial community structure—We examined the factors possibly controlling the relative abundance of the three groups, alpha- and beta-proteobacteria and *Actinobacteria,* that varied significantly along the estuarine gradient. All three groups correlated strongly with salinity ($r > 0.6$), but the relationships between their relative abundance and salinity varied (Fig. 5).

Alpha- and beta-proteobacteria had quite different relationships with salinity, both in sign and in the nature of the correlation. Alpha-proteobacterial abundance was lowest in freshwater and highest in marine waters (Fig. 5A), but the correlation between abundance and salinity was less strong when the freshwater values (salinity < 1) were excluded ($r = 0.40$, $p = 0.01$; $n = 40$) and insignificant when samples with salinity > 5 are considered ($r = 0.13$, $p > 0.05$; $n = 28$). Beta-proteobacterial abundance, in contrast, decreased throughout the salinity gradient, and the correlation with salinity remained high even if freshwater values were excluded ($r = -0.53$, $p < 0.0005$; $n = 40$). However, the abundance of this group varied about fourfold in freshwaters alone (Fig. 5B).

Like the beta-proteobacteria, *Actinobacteria* decreased with salinity (Fig. 5C). Again like beta-proteobacteria, actinobacterial abundance varied nearly eightfold in the freshwaters in the absence of a salinity gradient. Yet in spite of these similarities, the abundances of beta-proteobacteria and *Actinobacteria* were not significantly correlated (Table 3). As with the two proteobacterial groups, salinity only par-
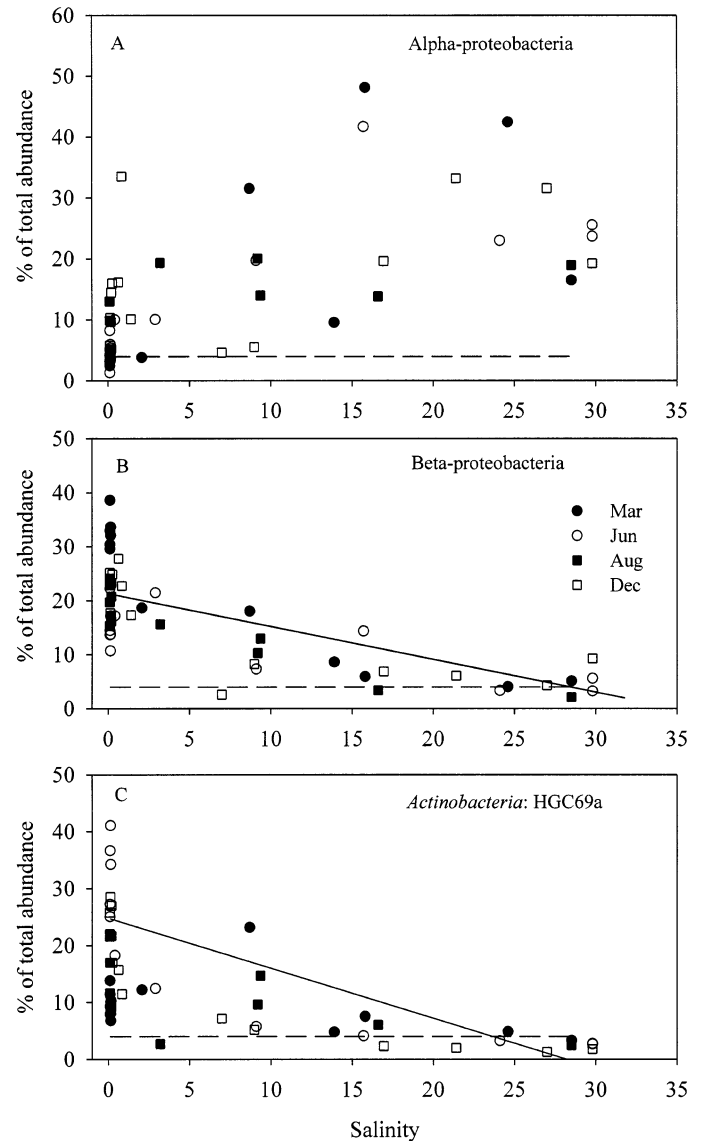

Fig. 5. Relationship between the relative abundance of (A) alpha-proteobacteria, (B) beta-proteobacteria, and (C) *Actinobacteria* recognized by the HGC69a probe and salinity. The solid line is the regression line. The dashed line is the negative control averaged over the entire estuary. Data from 2001 only are presented.

tially explains the variation in the abundance of *Actinobacteria.*

There were small but significant correlations between inorganic nitrogen concentrations and the abundances of alpha- and beta-proteobacteria, but there were no significant correlations with phosphate concentrations (Table 3). However, when the variation attributable to salinity was removed by partial correlation analysis, the correlations with inorganic nitrogen concentrations were not significant (data not shown).

Incorporation of thymidine and leucine by Actinobacteria—Thymidine and leucine incorporation by *Actinobacteria* was examined in more detail because the contribution of this

Table 3. Correlations for selected variables in the Delaware Estuary. The correlations were calculated for the entire data set consisting of measurements taken in 2001–2002 over the full length of the estuary. Abbreviations: Temp, temperature; Chl, chlorophyll *a*; Bact, bacterial abundance; TdR, thymidine incorporation rates; Leu, leucine incorporation rates; CF, *Cytophaga*-like bacteria; HGC69a, Actinobacteria recognized by the probe HGC69a as a percentage of total prokaryotic abundance. Alpha, beta, and gamma refer to the relative abundance (% of total abundance) of three subdivisions of Proteobacteria. Correlations greater than 0.6 are highlighted in italics. The number of samples was 51 for the HGC69a and 88 for the other measurements.

	Temp	Salinity	NH$_4$	NO$_3$	PO$_4$	Chl	Bact	TdR	Leu	Alpha	Beta	Gamma	CF	HGC69a
Temp	1													
Salinity	−0.04	1												
NH$_4$	−0.16	−0.50***	1											
NO$_3$	−0.02	*−0.75****	0.47***	1										
PO$_4$	0.03	−0.22	0.16	0.51***	1									
Chl	−0.17	0.04	−0.18	−0.17	−0.26	1								
Bact	0.453***	0.08	0.06	−0.30	−0.23	−0.17	1							
TdR	0.26	−0.54***	−0.04	0.14	0.04	−0.02	0.31*	1						
Leu	*0.60****	−0.05	−0.21	−0.15	−0.09	0.02	0.40*	*0.63****	1					
Alpha	0.01	*0.61****	−0.30	−0.30	−0.04	0.09	−0.10	−0.39*	−0.07	1				
Beta	−0.23	*−0.69****	0.37	0.44***	0.11	−0.16	−0.02	0.33*	−0.05	−0.37**	1			
Gamma	0.04	0.24	−0.26	0.07	0.16	0.02	−0.21	−0.14	−0.03	0.50***	−0.12	1		
CF	−0.07	0.31	−0.20	−0.17	−0.05	0.07	−0.01	−0.31	−0.14	0.35*	−0.21	0.23	1	
HGC69a	0.17	*−0.62****	0.08	0.34	−0.10	0.15	0.04	0.54***	−0.06	−0.42*	0.32	−0.15	−0.47*	1

* $p = 0.05/14 = 0.0036$, ** $p = 0.01/14 = 0.000714$, *** $p = 0.001/14 = 7.14 \times 10^{-5}$ (Bonferroni adjustment).

group to bacterial production has not been examined previously in the Delaware Estuary or elsewhere (Cottrell and Kirchman 2003). These bacteria were as active or more so as other bacteria in assimilating thymidine and leucine in the Delaware River (Table 4). The fraction of *Actinobacteria* assimilating thymidine and leucine was 28–40% and was generally as high as or higher than the fraction of all prokaryotes taking up these two compounds (20–37%; Table 4). The fraction of total silver grain area associated with *Actinobacteria* was equally high, ranging from 37% to 64%, depending on the compound and date, which was similar to or higher than the relative abundance of *Actinobacteria* (Table 4). These data suggest that the *Actinobacteria* are actively growing in the Delaware River and that their contribution to bacterial production is substantial when they are abundant, such as in the freshwaters of the estuary.

Diversity of the abundant bacterial groups in the estuary—We hypothesized that the lack of systematic variation in *Cytophaga*-like bacterial abundance was somehow related to the high diversity of this group. To examine this hypothesis, we calculated genetic distances among 16S rRNA sequences from the Delaware Estuary for the most abundant bacterial groups. In support of the hypothesis, the genetic distance among *Cytophaga*-like bacterial 16S rRNA sequences was significantly greater than the distance among alpha- and beta-proteobacteria and *Actinobacteria* in the Delaware River (Table 5). Similarly, the percent similarity of *Cytophaga*-like bacterial 16S rRNA sequences was on average significantly lower than for the other groups (Table 5). These data suggest that *Cytophaga*-like bacteria are more diverse than the other phylogenetic groups examined here.

It is more difficult to evaluate the hypothesis with data from the Delaware Bay. The general 16S rRNA library constructed by this study was so dominated by alpha-proteobacteria (98%) that it was not possible to examine the diversity of beta-proteobacteria and *Actinobacteria* in the Bay. The paucity of beta-proteobacteria and *Actinobacteria* in the Bay is consistent with the FISH data (*see above*), but *Cytophaga*-like bacteria were underrepresented in the library relative to the FISH data, as has been observed previously (Cottrell and Kirchman 2000b; Kirchman et al. 2003). Consequently, we evaluated the diversity of 16S rRNA genes from a library directed toward only *Cytophaga*-like bacteria in the Delaware Estuary (Kirchman et al. 2003). The diversity of these sequences was significantly higher than those of the alpha-proteobacteria in the River, but they were lower than of the *Cytophaga*-like sequences in the River and the alpha-proteobacteria sequences in the Bay (Table 5).

Discussion

Estuarine bacterial communities are potentially quite complex and could include groups normally found in freshwaters, oceanic waters, uniquely estuarine habitats (Crump et al. 2004), and perhaps even soils when runoff from land is high. Indeed, the Delaware Estuary does have all of the bacterial groups commonly found in freshwater and marine systems, including a group, *Actinobacteria,* not recognized previously as being abundant in estuaries. Terrestrial bacteria

Table 4. Assimilation of thymidine (TdR) and leucine (Leu) by prokaryotes and *Actinobacteria* recognized by probe HGC69a in June and December 2001. Samples were from the Delaware River, 200 km from the mouth of the estuary.

| | Total prokaryotes | | *Actinobacteria* | | % of total due to *Actinobacteria* | | | |
| | | | | | Abun-dance | SD | Assimila-tion** | SD |
Date	% active	SD*	% active	SD*				
June (TdR)	37.5	2.8	42.6	14	39	10	48	20
Dec (TdR)	19.1	3.0	28.5	6.4	31	10	65	16
Dec (Leu)	22.8	3.6	40.8	9.4	31	10	37	20

* Standard deviation of 30 microscopic fields of view.
** Assimilation was estimated from silver grain area.

do not appear to be abundant in this estuary. Analysis of clone libraries constructed with Delaware River samples did not find any 16S rRNA gene sequences, including those from *Actinobacteria,* closely related to those from terrestrial environments (Cottrell et al. in press). The *Actinobacteria* in seven freshwater lakes and reservoirs were also not related to soil bacteria (Warnecke et al. 2004). Although these bacterial groups are potentially complex and diverse, the abundance of alpha- and beta-proteobacteria and *Actinobacteria* varied consistently along the estuarine gradient regardless of the season.

There have been several studies of bacterial community structure in estuaries, but the work of Bouvier and del Giorgio (2002) is most similar to ours because the Chesapeake Bay is geographically close to the Delaware Bay and because Bouvier and del Giorgio (2002) used FISH, as in this study. Although both estuarine systems shared some general patterns (e.g., the switch between alpha- and beta-proteobacterial abundance), our results differ from those of Bouvier and del Giorgio (2002) in several respects. We suspect that some of the differences are due to differences in FISH methodology; we used Cy3-labeled probes, for example, whereas Bouvier and del Giorgio (2002) used Alexa labeling. This and other methodological differences probably explain why the relative abundances of the bacterial groups appear to be higher in the Delaware than in the Chesapeake

Bay estuaries examined by Bouvier and del Giorgio (2002). In samples from the main stem of the Chesapeake Bay, we found relative abundance of these bacterial groups to be similar to the Delaware Bay at comparable salinities (results not shown).

Bouvier and del Giorgio (2002) hypothesized that the changes in bacterial community composition in the Chesapeake Bay estuaries they examined were due to various "bottom up" factors affecting bacterial growth. One factor they identified was physiological stress caused by the mixing of low- and high-salinity waters in the turbidity maximum zone of the estuary. In support of their hypothesis, they found that bacterial growth efficiency was lower in the turbidity maximum region in which the abundance of Eub338-positive cells was also low, which Bouvier and del Giorgio (2002) suggested was due to low activity (low ribosome content) of these bacteria. We also observed that the relative abundance of the Eub338-positive cells tended to be lower in the transition zone of the Delaware Estuary, although this fraction varied much less (45–70%, on average) than observed in the Chesapeake Bay estuaries (5–80%). We suspect that the lower Eub338 signal in the Delaware transition zone was due in part to methodological problems caused by the high turbidity of this region. Furthermore, there was no significant relationship between relative Eub338-positive cell

Table 5. Diversity of bacterial groups abundant in the Delaware Estuary. The "River" and "Bay" samples were from 200 km and 10 km from the mouth of the estuary, respectively. The 16S rRNA sequences for the River sample were taken from Cottrell et al. (in press). The Bay *Cytophaga* data are from Kirchman et al. (2003), whereas the bay alpha-proteobacteria data were collected for this study. "All" refers to all ribotypes that match the Alfa968a probe for alpha-proteobacteria and the CF319a probe for *Cytophaga*-like bacteria in the RDP 16S rRNA data base.

Location	Bacterial group	Distance	Similarity 95% CI*	(%)	95% CI	n**
River	Alpha-proteobacteria	0.008	0.007	99.2	0.7	10
River	Beta-proteobacteria	0.129	0.005	88.4	0.4	741
River	*Actinobacteria*	0.097	0.010	91.1	0.8	171
River	*Cytophaga*-like	0.173	0.005	84.9	0.4	1,035
Bay	Alpha-proteobacteria	0.173	0.036	85.2	2.9	36
Bay	*Cytophaga*-like	0.092	0.021	91.5	1.9	21
All	Alpha-proteobacteria	0.164	0.0001	85.4	0.01	877,150
All	*Cytophaga*-like	0.217	0.0006	81.4	0.02	52,003

* 95% confidence interval.
** *n* is the number of comparisons.

abundance and measures of microbial activity in the Delaware Estuary.

Other bottom-up factors explored by Bouvier and del Giorgio (2002) included inorganic and organic nutrient concentrations. In the Chesapeake Bay system, the abundance of alpha-proteobacteria was negatively correlated with phosphate concentrations, whereas beta-proteobacterial abundance was positively correlated with dissolved organic carbon (DOC) concentrations. Kirchman et al. (2004) found a positive correlation between beta-proteobacterial abundance and phosphatase activity in the Hudson River. We did not find any correlations between community structure and phosphate and DOC concentrations, and the weak relationships with inorganic nitrogen concentrations proved to be insignificant when salinity effects were removed. Indeed, community structure seemed independent of several bottom-up parameters that often affect bulk bacterial biomass and growth in the Delaware Estuary and other aquatic habitats. Previous studies of freshwater systems have also noted that bottom-up factors do not completely explain bacterial community structure (Gasol et al. 2002; Simek et al. 2003).

One biogeochemical parameter, salinity, did affect community structure. The relative abundance of *Actinobacteria* and beta-proteobacteria both correlated negatively and strongly with salinity, suggesting that these bacteria grew in the Delaware River and then were mixed conservatively with coastal waters containing low numbers of beta-proteobacteria and *Actinobacteria.* Consistent with this hypothesis, the fraction of beta-proteobacteria actively incorporating thymidine was high in freshwater and low in the marine waters of the Delaware Bay (Cottrell and Kirchman 2004), and growth rates of bacteria in this group were higher in the Delaware River than in the Bay (Yokokawa et al. 2004). Still, although salinity might explain some of this variation, the relative abundance of beta-proteobacteria and of *Actinobacteria* varied nearly as much over the seasons in the Delaware River (zero salinity) as it did in the rest of the estuary.

Actinobacterial abundance might be underestimated in the Delaware Bay because the FISH probes we used were based on freshwater phylotypes (Glöckner et al. 2000). However, we found only one actinobacterial 16S rRNA clone among the 89 clones we screened in a library from the coastal end member of the estuary. Other clone library data also suggest that the abundance of *Actinobacteria* is <10% of total prokaryotic abundance in coastal and oceanic waters (Acinas et al. 2004; Venter et al. 2004).

In contrast to beta-proteobacteria and *Actinobacteria,* alpha-proteobacteria were not abundant in the Delaware River, but they often dominated bacterial communities of the Bay. Unlike beta-proteobacteria and *Actinobacteria,* the relationship between alpha-proteobacterial abundance and salinity was weak, suggesting that salinity has a secondary effect on the growth of these bacteria. In fact, alpha-proteobacteria are active in incorporating thymidine and leucine in both the Delaware River and the Bay (Cottrell and Kirchman 2004), and growth rates of alpha-proteobacteria can be high in both fresh- and marine waters of the estuary (Yokokawa et al. 2004). These data indicate that some aquatic alpha-proteobacteria are capable of growth in freshwaters.

Cytophaga-like bacteria were often quite abundant in both the Delaware River and Bay, but their abundance did not vary systematically along the estuarine gradient, although they tended to be most abundant at the marine end of the estuary. Similar to previous studies, in some months, the abundance of *Cytophaga*-like bacteria was higher in the turbidity maximum zone (Cottrell and Kirchman 2003), but this pattern was not consistently observed in all months and there was no correlation with attenuation (data not shown). Overall, the abundance of the other dominant bacterial groups (alpha- and beta-proteobacteria and the *Actinobacteria*) in the estuary varied much more systematically than did *Cytophaga*-like bacterial abundance. There is some evidence for two complementary hypotheses that explain why three bacterial groups varied systematically whereas *Cytophaga*-like bacteria did not.

One hypothesis is that, compared with the other abundant bacterial groups, more members of the *Cytophaga*-like bacterial group are physiologically plastic enough to flourish throughout the estuarine gradient. In fact, some *Cytophaga*-like bacterial ribotypes, such as members of Delaware Cluster 2, are found throughout the Delaware Estuary (Kirchman et al. 2003; Castle and Kirchman 2004). The success of *Cytophaga*-like bacteria is consistent with data indicating that polysaccharides and chitin, which *Cytophaga*-like bacteria are thought to use proficiently (Kirchman 2002), are found throughout the Delaware Estuary (Kirchman and Borch 2003; Kirchman and White 1999). It is unclear whether ribotypes from other bacterial groups occur throughout the estuary because the 16S rRNA sequence data are sufficient only for *Cytophaga*-like bacteria for the entire estuary. It is true that SAR11 bacteria were found in both the Delaware River and Bay, and relatives of this abundant marine group have been found even in freshwater lakes (Bahr et al. 1996). However, overall, few bacteria appear capable of growing throughout the entire estuarine gradient (Troussellier et al. 2002; Crump et al. 2004).

The other hypothesis is that the phylogenetic diversity of *Cytophaga*-like bacteria is higher than that of the other bacterial groups and that this diversity is associated with phenotypic diversity, which enabled the *Cytophaga*-like group to be abundant throughout the estuary. A diverse group having many different ribotypes, each adapted to a particular region of the estuary, would be more abundant overall than a less diverse bacterial group. Data on 16S rRNA sequences provide some support for the diversity hypothesis. The 16S rRNA genes of *Cytophaga*-like bacteria from the Delaware River were significantly more diverse than the other bacterial groups in the river. The Bay data are inadequate for testing this hypothesis because of the lack of sequences for beta-proteobacteria and *Actinobacteria.* Also, we wonder whether the *Cytophaga*-directed library captured the diversity of these bacteria in the bay because the 16S rRNA genes from this library were substantially less diverse than the *Cytophaga* 16S rRNA genes in the general libraries from the River.

In any case, a more global analysis is needed to test whether the diversity hypothesis explains the biogeographical patterns observed in lakes and oceans as well as estuaries. To begin to explore this hypothesis, we compared the diversity of all alpha-proteobacteria and *Cytophaga*-like bac-

teria in the data base. We used ARB (Ludwig et al. 2004) to find all ribotypes in the August 2002 version of the aligned Ribosomal Database Project (Maidak et al. 2001) that were recognized by the FISH probes for *Cytophaga*-like bacteria (CF319a) or for alpha-proteobacteria (Alf968). A ribotype was included in one of the two groups if the respective probe bound perfectly (no mismatches) to it. This analysis showed that *Cytophaga*-like bacteria are significantly more diverse than the alpha-proteobacteria (Table 5). Data from other studies also support the hypothesis that *Cytophaga*-like bacteria are more diverse than alpha-proteobacteria in the ocean (Acinas et al. 2004) and in estuaries (Crump et al. 2004).

The relationship between phenotypic diversity and phylogenetic diversity affects how we use bacterial groups to examine biogeochemical cycles. The presence of systematic biogeographical patterns for some groups, even those at a high phylogenetic level, indicates that they might function as ecological units with defined roles in mediating biogeochemical processes. In support of this hypothesis, there has been some success in explaining DOC degradation at the subdivision level (Cottrell and Kirchman 2000b; Schweitzer et al. 2001; Kirchman et al. 2004), and Horner-Devine et al. (2003) found it informative to examine groups at this phylogenetic level for exploring relationships between bacterial diversity and chlorophyll concentrations in freshwater mesocosms. This work also indicates the need for further study of the macroevolution of microbes (evolution above the species level), a rich topic in macroorganismal evolution but neglected in studies of microbial evolution (Martin et al. 2004). On the other hand, the lack of systematic biogeographical patterns for *Cytophaga*-like bacteria point to the need to find ecologically relevant subgroups of these bacteria, although some generalizations applicable for the entire *Cytophaga*-like group have been made (Kirchman 2002). The appropriate phylogenetic level for exploring links between bacterial community structure and function will probably depend on the ecological question and the bacterial group.

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