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Natural Assemblages of Marine Proteobacteria and Members of the *Cytophaga-Flavobacter* Cluster Consuming Low- and High-Molecular-Weight Dissolved Organic Matter

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We used a method that combines microautoradiography with hybridization of fluorescent rRNA-targeted oligonucleotide probes to whole cells (MICRO-FISH) to test the hypothesis that the relative contributions of various phylogenetic groups to the utilization of dissolved organic matter (DOM) depend solely on their relative abundance in the bacterial community. We found that utilization of even simple low-molecular-weight DOM components by bacteria differed across the major phylogenetic groups and often did not correlate with the relative abundance of these bacterial groups in estuarine and coastal environments. The *Cytophaga-Flavobacter* cluster was overrepresented in the portion of the assemblage consuming chitin, *N*-acetylglucosamine, and protein but was generally underrepresented in the assemblage consuming amino acids. The amino acid-consuming assemblage was usually dominated by the α subclass of the class *Proteobacteria*, although the representation of α-proteobacteria in the protein-consuming assemblages was about that expected from their relative abundance in the entire bacterial community. In our experiments, no phylogenetic group dominated the consumption of all DOM, suggesting that the participation of a diverse assemblage of bacteria is essential for the complete degradation of complex DOM in the oceans. These results also suggest that the role of aerobic heterotrophic bacteria in carbon cycling would be more accurately described by using three groups instead of the single bacterial compartment currently used in biogeochemical models.

Analysis of 16S rRNA gene sequences (15) has greatly advanced our understanding of the phylogenetic diversity of bacteria and archaea (18), especially that of the vast majority of microbes in nature that have resisted cultivation to date (2). There is little information, however, on the metabolic function of specific bacterial groups in natural assemblages since few culture-independent studies have linked bacterial community structure and function (6). Although information on phylogenetic relationships of uncultured bacteria is readily accessible (14), the inability to culture most microbes limits the opportunities to assess their metabolic diversity. Even if appropriate culture conditions were to be found for the bulk of marine microbes, bacterial metabolism in the sea would probably remain poorly described, since metabolic behavior in culture is likely different from that in situ.

Extensive biogeochemical studies have shown that uptake and mineralization of dissolved organic matter (DOM) by bacteria constitute a major component of carbon cycling in aquatic ecosystems (11). Although the importance of DOM uptake is well recognized, the relative contributions of the major phylogenetic groups of bacteria to DOM uptake in the oceans are unknown (29). Differences in usage of various DOM components may help explain the distribution of the major bacterial groups among soil, freshwater, and marine ecosystems (18). It may also be important to know the minimum number of bacterial phylogenetic groups necessary to describe and explain DOM uptake in order to improve models of carbon cycling in aquatic habitats. Currently these models implicitly assume that all heterotrophic bacteria are the same and consist of a single phylogenetic type (12).

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The goal of this study was to determine whether the relative contributions of various phylogenetic groups to the utilization of DOM depend solely on their relative abundance in the bacterial community. We used a novel approach, combining microautoradiography and fluorescence in situ hybridization (MICRO-FISH) (22, 28) to determine DOM uptake by the bacterial divisions and subclasses typically comprising marine assemblages (16). Since the chemical composition and degradation of DOM differ as a function of molecular weight (3, 4), different groups of bacteria may be responsible for mineralizing low- and high-molecular-weight DOM. We hypothesized that all heterotrophic bacteria use low-molecular-weight DOM, specifically monomers that can be transported easily across cell membranes. High-molecular-weight DOM, on the other hand, may be consumed by a smaller, less-diverse group of bacteria since specific extracellular enzymes are required for the hydrolysis of biopolymers, a component of high-molecular-weight DOM. To test these hypotheses, we used MICRO-FISH to compare utilization of protein and chitin by various phylogenetic groups with their utilization of amino acids and *N*-acetylglucosamine (NAG). Protein and chitin were chosen because they represent potentially large components of high-molecular-weight DOM (25).

Sample collection and incubation. Seawater was collected from the Delaware Bay estuary at the Roosevelt Inlet (salinity [S] = 30 ppt, temperature [T] = 14°C) and from the Atlantic Ocean at the Indian River Inlet (S = 32 ppt, T = 12°C) in November and December, respectively. Aliquots were incubated at 12 to 19°C and tritiated amino acids, NAG, protein, and chitin were added. The final concentrations of the amino acid mixture (47 Ci/mmol; Amersham) and NAG (9.9 Ci/mmol; Amersham) additions were 2.1 and 10 nM, respectively. Soluble chitin oligomers were prepared by mild acid hydrolysis (3 N HCl, 70°C, 5 min) of tritiated chitin purified from the marine fungus *Paeosphaeria spartinicola* (27) grown on me-

dium containing [^{3}H]NAG (21). Tritiated protein was prepared from *Vibrio alginolyticus* grown on medium containing [^{3}H]leucine (26). Subsamples were filtered through 0.2-μm-pore-size polycarbonate filters to measure the uptake of radiolabeled compounds in incubations lasting 1 to 26 h. Amino acid incubations were for 1 h, the protein incubation with the assemblage from Roosevelt Inlet lasted 26 h, and the remaining incubations were for 7 h. Bacterial abundance was measured by determining 4′,6′-diamidino-2-phenylindole (DAPI) direct counts (30).

MICRO-FISH. Samples were prepared for MICRO-FISH by using a variation of methods combining microautoradiography and fluorescence in situ hybridization. Unlike substrate-tracking autoradiographic fluorescent in situ hybridization (28), but similar to the protocol of Lee et al. (22), cells were transferred to glass coverslips and probed with fluorescent oligonucleotides before being coated with an autoradiographic film emulsion. After incubation with tritiated compounds, samples were fixed with formaldehyde, subsamples for MICRO-FISH were filtered through 0.2-μm-pore-size polycarbonate filters, and the cells were transferred to glass coverslips freshly treated with a 2% solution of 3-aminopropyltriethoxysilane (Sigma) (5). Immediately after filtration, the polycarbonate filter was placed face down on a coverslip, clamped together between two glass slides by the use of a large paper clip, and incubated for 1 h at 42°C. The filter was then peeled away, and the cells were dehydrated by passing the coverslip through a series of ethanol rinses and then air dried. Unlike the protocol of Lee et al. (22), our method does not require a special slide with a hole for viewing cells attached to the back of the coverslip.

In situ hybridization was done by placing the cell-adherent side of the coverslip in contact with a 30-μl drop of hybridization solution containing 2 ng of probe/μl in the bottom of a polystyrene petri dish. The probes used were as follows: for bacteria, Eub338 (positive control) (1); for α-proteobacteria, Alf1b (24); for β-proteobacteria, Bet42a (24); for γ-proteobacteria, Gam42a (24); for the *Cytophaga-Flavobacter* cluster of the *Cytophaga-Flavobacter-Bacteroides* division, CF319a (23); and for gram-positive bacteria with high DNA G+C content, HGC69a (32). The dish was sealed with Parafilm and incubated at 42°C for 2 h, after which the coverslip was incubated for 30 min at 48°C in a wash solution containing NaCl at a concentration appropriate for the probe (34). The coverslip was then rinsed in deionized water, air dried, and mounted (by the use of immersion oil) on a glass slide with the cell-adherent side of the coverslip facing away from the slide.

Samples were prepared for microautoradiography by dipping the glass slide, with coverslip attached, into a molten (43°C) NBT-2 emulsion (Kodak) diluted to 2 parts emulsion and 1 part deionized water. After incubation at −20°C for 2 days, the slides were warmed to room temperature and the photographic emulsion was developed by using Dektol developer (Kodak), a deionized-water stop bath, and fixer (Kodak) in accordance with the manufacturer's instructions. The slide was stained in a 2-μg/ml solution of DAPI for 2 min, dipped in deionized water, and air dried. The coverslip was removed from the glass slide and mounted on a clean glass slide with the cells facing the slide, using Citifluor (Ted Pella Inc., Redding, Calif.). Cells were examined by using a fluorescence microscope fitted with filter sets for DAPI (UV1A; Nikon) and Cy3 (41007A; Chroma). The average level of retention of cells on the coverslip through all steps of the procedure was 103%, as determined by DAPI counts of bacteria on black polycarbonate filters (30).

Phylogenetic groups consuming DOM compounds. MICRO-FISH identifies bacteria that have taken up tritiated compounds by determining the presence of silver grains adjacent to cells (Fig. 1). The phylogenetic classification of cells is determined by the binding of rRNA-targeted oligonucleotide probes conjugated to the yellow-fluorescing fluorochrome Cy3 (Fig. 1B). The sample shown in Fig. 1 was prepared by using tritiated amino acids typically consumed by a large fraction of cells (10, 19) and a positive-control probe complementary to a region of the 16S rRNA conserved in most bacteria (Eub338) (1), so all bacteria consuming free amino acids and having sufficient numbers of ribosomes are visible. The eubacterial probe detected on average 80% (standard deviation, 9.0) of the bacterial abundance determined by DAPI direct counts.

Bacterial assemblages in Delaware estuarine and coastal waters were dominated by proteobacteria and members of the *Cytophaga-Flavobacter* cluster (Fig. 2A and 3A). Proteobacteria, and to a lesser extent the *Cytophaga-Flavobacter* cluster, are typically abundant in aquatic systems (31, 33). Three subclasses of proteobacteria (α, β, and γ) were about equally abundant in the coastal sample, while α-proteobacteria were most abundant in the estuarine sample. The relatively large abundance of members of the *Cytophaga-Flavobacter* cluster may be a consequence of high particle loads in these environments; studies inferring community composition from libraries of cloned 16S rRNA genes amplified by PCR have found that this group is enriched on particles (9). However, Glöckner et al. (16), using fluorescence in situ hybridization, found that a large abundance of bacteria in the *Cytophaga-Flavobacter* cluster may be common in marine systems. Cells binding the probe for gram-positive bacteria accounted for less than 3% of the direct count, which is not significantly different from counts of autofluorescent cells in controls without a probe. The probes for α-, β-, and γ-proteobacteria and the *Cytophaga-Flavobacter* cluster detected 70% (standard deviation, 30) of the bacteria visualized with the control probe (Eub338) for all bacteria (Fig. 2A and 3A).

Consumption of organic compounds differed among the phylogenetic groups. Even uptake of low-molecular-weight DOM differed greatly among groups. The *Cytophaga-Flavobacter* cluster accounted for the largest fraction of bacteria consuming chitin, NAG, and protein but was the smallest fraction consuming amino acids (Fig. 2B and Fig. 3B). In contrast, α-proteobacteria comprised the largest fraction of the community consuming amino acids but the smallest fraction consuming protein. Differences between amino acid consumption by α-proteobacteria and by members of the *Cytophaga-Flavobacter* cluster occurred in both estuarine and coastal environments (Fig. 2B and 3B), but consumption of amino acids by these two groups in the San Pedro Channel off the California coast did not differ (28). In the San Pedro Channel, these two groups were equally abundant and about 80% of the cells in each group actively took up amino acids.

Although a large (>50%) fraction of bacteria sometimes could not be identified with the four group-specific probes used in this study (Fig. 2A and 3A), usually only a small fraction (<20%) of the bacteria actively taking up the various ^{3}H-labeled compounds remained unidentified (Fig. 2B and 3B). The single exception occurred in the estuarine experiment examining chitin utilization. Nearly 40% of the bacteria assimilating ^{3}H-chitin oligomers could not be assigned to one of the four phylogenetic groups examined (Fig. 2B).

There was no fixed relationship between utilization of polymers and their constituent monomers by different phylogenetic groups. The same phylogenetic groups accounted for most of the cells consuming chitin and NAG, but protein and amino

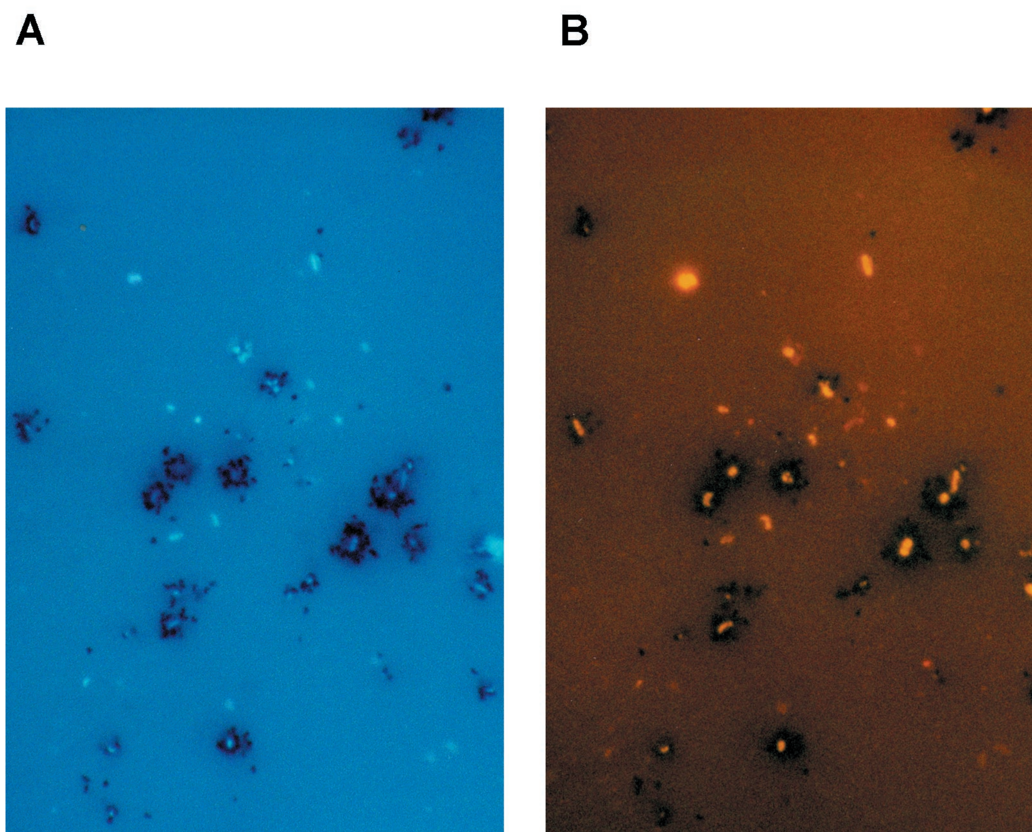

FIG. 1. Micrograph of bacteria assayed by MICRO-FISH. (A) DAPI-stained bacteria (UV excitation). Dark spots surrounding cells are silver grains deposited in photographic emulsion around cells that took up a mixture of tritiated free amino acids. Less than 0.6% of cells in formaldehyde-killed controls had silver grains. (B) Bacteria hybridized with Cy3-labeled oligonucleotide probe Eub338 for eubacteria (green excitation). Cells with bound probe fluoresce yellow. Magnification, ×1,350.

acids were largely consumed by different phylogenetic groups. Members of the *Cytophaga-Flavobacter* cluster accounted for 30 and 47% of the community consuming chitin and NAG, respectively, and α-proteobacteria accounted for 22 and 45% of the cells consuming these compounds, respectively. In contrast, the *Cytophaga-Flavobacter* cluster accounted for 45% of the cells consuming protein but only 3% of the cells consuming amino acids, while α-proteobacteria accounted for 45% of the cells consuming amino acids but only 10% of the cells consuming protein.

Composition and activity of bacterial assemblages. The distributions of β- and γ-proteobacteria are among the most striking features of microbial diversity in aquatic environments. Although these two groups coexist in coastal environments (31), β-proteobacteria are not found in the oligotrophic ocean but are abundant in freshwater habitats, where they seem to displace γ-proteobacteria (16, 17). Variations in the supply and composition of DOM in freshwater versus marine systems (3, 13) may determine the distribution of β- and γ-proteobacteria if these two groups differ in the capacity to utilize various DOM components. Our hypothesis is based on the observation that growth of the total bacterial community is often limited by the availability of DOM (7, 8, 20). An alternative hypothesis is that β- and γ-proteobacteria use the same compounds present in both oceanic and freshwater environments and that β-proteobacteria are restricted from the oligotrophic ocean by a selection factor other than DOM.

Our results suggest that β- and γ-proteobacteria are similar with respect to DOM consumption. These two groups comprised the smallest fraction (15% or less) of bacteria consuming chitin and NAG, and they accounted for 19 to 29% of the cells consuming protein and amino acids. This analysis suggests that the availability of DOM does not explain the distributions of β- and γ-proteobacteria in aquatic environments.

The relative abundance of phylogenetic groups of bacteria in assemblages consuming various DOM components often differed from their relative abundance in the assemblage as a whole. The *Cytophaga-Flavobacter* cluster was overrepresented in the portion of the assemblage consuming chitin (Fig. 4A), NAG (Fig. 4B), and protein (Fig. 4C). Among the cells identified by the bacterial probe (Eub338), the *Cytophaga-Flavobacter* cluster comprised 23 to 55% of the cells consuming chitin, NAG, and protein, but these bacteria made up only 8 to 38% of the assemblage (Fig. 4A to C). In contrast, the *Cytophaga-Flavobacter* cluster was greatly underrepresented in the assemblage consuming amino acids (Fig. 4D), accounting for only 2 to 4% of the amino acid-consuming bacteria. All three subclasses of proteobacteria were equally or underrepresented among bacteria consuming chitin (Fig. 4A), but their participation in protein usage was less clear. Protein use by α-proteobacteria was about that expected from their relative abundance in the total bacterial community, while β- and γ-proteobacteria were over and underrepresented among the bacteria consuming protein in the two environments sampled in this study (Fig. 4C). The β and γ subclasses of proteobacteria comprised a small portion of the assemblage (<10%)

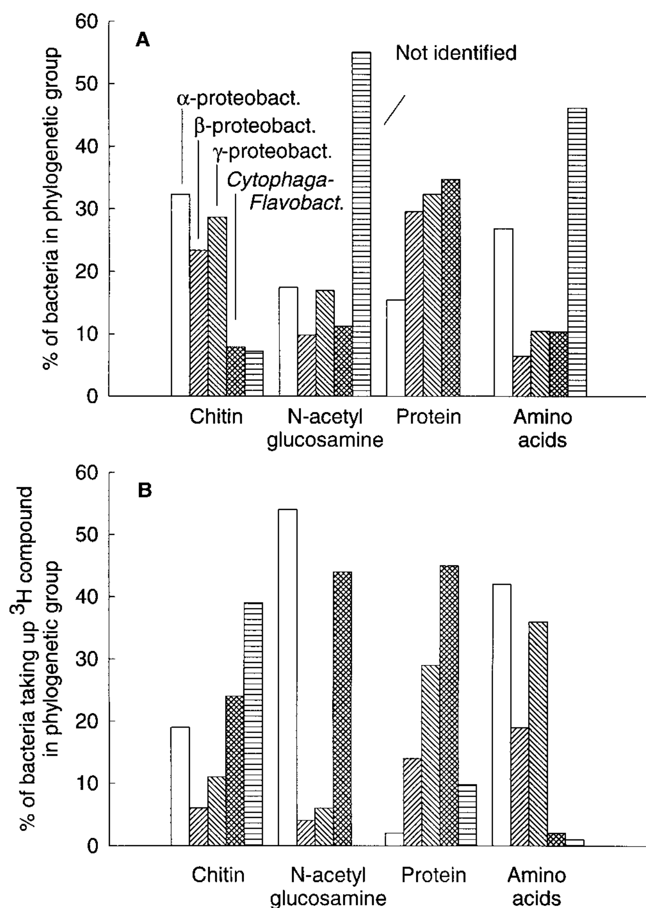

FIG. 2. Community composition and consumption of chitin, NAG, protein, and amino acids by the major phylogenetic groups of bacterioplankton in the Roosevelt Inlet, assayed by MICRO-FISH. (A) Composition of bacterioplankton communities in incubations containing tritiated compounds. (B) Relative abundance of phylogenetic groups of bacteria consuming various tritiated compounds. Less than 3% of the cells were gram positive. Cells binding none of the group-specific probes are indicated (Not identified). Percentages were calculated relative to total bacteria counted by using DAPI, although the eubacterial probe (Eub338) detected on average 80% of bacterial abundance. proteobact., proteobacteria; *Flavobact.*, *Flavobacter*.

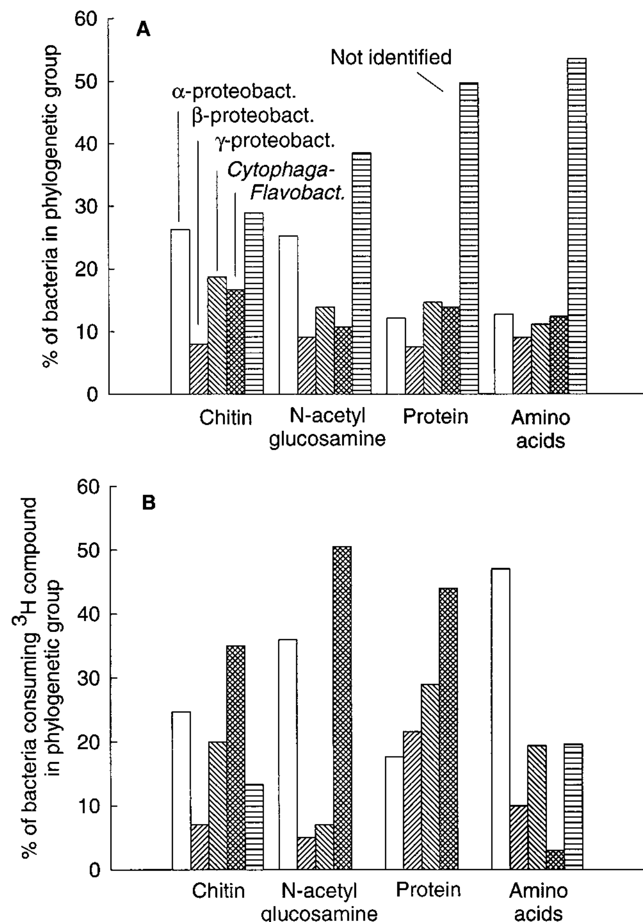

FIG. 3. Community composition and consumption of chitin, NAG, protein, and amino acids by the major phylogenetic groups of bacterioplankton in the Indian River Inlet, assayed by MICRO-FISH. (A) Composition of bacterioplankton communities in incubations containing tritiated compounds. (B) Relative abundance of phylogenetic groups of bacteria consuming various tritiated compounds. Less than 3% of the cells were gram positive. Cells binding none of the group-specific probes are indicated (Not identified). Percentages were calculated relative to total bacteria counted by using DAPI, although the eubacterial probe (Eub338) detected on average 80% of the bacterial abundance.

consuming NAG relative to their abundance (10 to 25%) (Fig. 4B). Uptake of amino acids differed greatly among the three subclasses of proteobacteria and between experiments. The percentage of amino acid-consuming bacteria that were proteobacteria sometimes was greater than, equal to, and less than their contribution to total bacterial abundance (Fig. 4D).

Changes in community structure due to protein addition were consistent with the MICRO-FISH results. Addition of protein in the Roosevelt Inlet experiment caused a large increase in the abundance of the same bacterial groups revealed by MICRO-FISH to consume protein. α-Proteobacteria initially dominated the community (27% of the total), but after incubation with protein, bacteria in the *Cytophaga-Flavobacter* cluster were the most abundant (35% of the community) and α-proteobacteria were the least abundant (Fig. 2A). MICRO-FISH indicated that 45% of the cells consuming protein were members of the *Cytophaga-Flavobacter* cluster and that only 2% were α-proteobacteria (Fig. 2B). *Cytophaga-Flavobacter* cluster members did not dominate protein consumption simply because they grow rapidly in bottle incubations while α-proteobacteria grow slowly. In a shorter incubation with an assemblage from the Indian River Inlet, there was no shift in the community composition (Fig. 3A). MICRO-FISH again revealed that members of the *Cytophaga-Flavobacter* cluster accounted for most of the cells consuming protein and that α-proteobacteria accounted for the smallest fraction (Fig. 3B).

In revealing the differences in DOM uptake by the various heterotrophic bacteria, this study indicates the need to consider more than a single compartment for modeling the role of heterotrophic bacteria in carbon cycles. However, our results also suggest that models may not require inclusion of the entire diverse spectrum of organisms found by culture-independent studies (15, 18). We found that, with one exception, all of the bacteria assimilating DOM components could be assigned to one of the four phylogenetic groups examined, although other bacterial groups undoubtedly assimilate some DOM. Furthermore, since β-proteobacteria are not commonly found in oceans (16, 18), and in any case their activity seems similar to

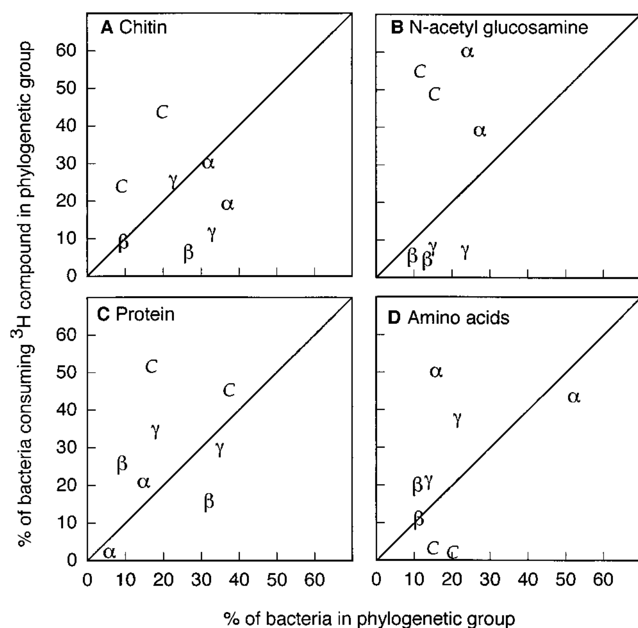

FIG. 4. Relationship among the phylogenetic classifications of bacteria consuming chitin (A), NAG (B), protein (C), and amino acids (D) versus phylogenetic classification of cells identified as eubacteria. Bacteria were classified by using rRNA-binding oligonucleotide probes specific for α-proteobacteria (α), β-proteobacteria (β), γ-proteobacteria (γ), and the *Cytophaga-Flavobacter* group (*C*). Data points falling above the 1:1 line indicate phylogenetic groups enriched in the portion of the assemblage consuming the compound. Results are from coastal (Fig. 2) and estuarine (Fig. 3) environments. Percentages were calculated relative to the numbers of cells identified as eubacteria with the Eub338 probe.

that of γ-proteobacteria, it appears that uptake of DOM may be explained by three bacterial groups, with properties represented by α- and γ-proteobacteria and the *Cytophaga-Flavobacter* cluster. Our generalization about DOM uptake, however, may not apply to other environments (e.g., soils), where these three phylogenetic groups probably have different metabolic capacities.

In general, the number of groups required to describe relationships between bacterial community structure and function is unclear. The phylogenetic level on which to focus is also not obvious. In our study, we found that consumption of DOM could be explained using a relatively small number of phylogenetic groups (at most four) at the division and subclass levels. Understanding other structure-function relationships, however, may require examining a larger number of more closely related phylogenetic groups. For example, temporal shifts in DOM consumption might be explained at the family or genus level, and more bacterial groups may be necessary for explaining DOM uptake in aquatic environments (e.g., oligotrophic oceans) that differ greatly from the eutrophic waters of our study. Although more data are clearly needed, our study suggests that comparing DOM consumption across environments at the division and proteobacterial-subclass levels will enhance our understanding of this structure-function relationship in marine bacterial communities.

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