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Changes in bacterial β-glucosidase diversity during a coastal phytoplankton bloom

Abstract—Bacterial enzymatic hydrolysis of high molecular weight organic matter is the rate-limiting step in the bacterially mediated carbon cycling in the global ocean. Despite the importance of this process, only bulk measurements of these hydrolytic activities are available, and the dynamics and diversity of the ectohydrolases involved in the cleavage of high molecular weight organic matter are poorly understood. In this study we monitored the dynamics of bacterial β-glucosidase diversity during the wax and wane of a coastal phytoplankton bloom using a newly developed capillary electrophoretic assay. Up to eight different β-glucosidases were detected in a single sample and 11 over the whole study period, revealing a previously unnoticed β-glucosidase diversity. A close link was found between the temporal succession of β-glucosidase diversity and bacterioplankton species richness as determined by terminal-restriction fragment length polymorphism analysis. This indicates that the regulation of the β-glucosidase activity and diversity was driven by shifts in the bacterial community structure rather than by simple induction of enzyme expression within a stable bacterioplankton community.

Bacterioplankton are the only significant consumers of the oceanic dissolved organic matter (DOM) pool. Most of the bioavailable DOM is of high molecular weight (Amon and Benner 1994, 1996) and has to be cleaved by hydrolytic enzymes outside the cell before it can be taken up by bacteria (Payne 1980; Smith et al. 1992). Since polysaccharides are the most abundant class of chemically identifiable compounds of the oceanic DOM pool, bacterial β-glucosidase is one of the key hydrolases for marine bacteria (Hoppe et al. 1990; Smith et al. 1995). Bacterial β-glucosidase activity is ubiquitously present in aquatic systems, but its intensity and kinetic characteristics are subjected to large spatial and temporal variations. The synthesis of β-glucosidases is triggered by the presence of suitable substrate and inhibited by readily available monomeric compounds (Chróst 1991; Middelboe et al. 1995).

All the previous work has been based on bulk measurements of bacterial hydrolytic activity and assumes a homogeneous type of enzyme acting on a given substrate despite of the quite heterogeneous bacterial communities found in marine systems. However, the factors stimulating bacterial hydrolytic activities such as phytoplankton blooms also promote changes in the bacterial community structure (Pinhassi and Hagström 2000) and not all the marine bacteria produce significant amounts of β-glucosidase (Martinez et al. 1996; Herndl et al. 1999). In the only study on the diversity of β-glucosidases in marine systems we are aware of (Rath and Herndl 1994), only one to two types of β-glucosidase were found in spite of the high species diversity commonly reported for marine bacterial communities (Giovannoni and Rappé 2000). Rath and Herndl (1994) suggested that β-glucosidase activity is either expressed by only a few specialized bacteria in marine systems or, alternatively, that a family of β-glucosidase genes has spread by horizontal gene transfer among marine bacteria. Chitinase genes of marine bacteria belonging to distant phylogenetic groups such as the α- and γ-proteobacteria have been recently found to be closely related (Cottrell et al. 1999, 2000), supporting the idea of the importance of horizontal gene transfer. However, the chitinases of other marine bacterial groups such as the *Cytophaga-Flavobacter* group were different from those of α- and γ-proteobacteria (Cottrell et al. 1999).

The distribution of organic matter in marine systems is thought to be rather heterogeneous on a microspatial scale (Azam 1998). Consequently, the substrate concentration on a microscale may vary over several orders of magnitude in the different dissolved, colloidal, and particulate fractions of the organic matter pool. It is therefore unlikely that a single type of β-glucosidase can effectively operate over this large range of possible substrate concentrations. In fact, kinetic studies of enzyme activity in aquatic systems often reveal biphasic or multiphasic enzyme kinetics, suggesting the coexistence of different β-glucosidases (Talbot and Bianchi 1997; Tholosan et al. 1999; Unanue et al. 1999).

The purpose of this study was to characterize the diversity of β-glucosidases in a coastal marine system and to determine the dynamics of these β-glucosidases during to the development and collapse of a coastal phytoplankton bloom.

Materials and methods—Bacterial ectoenzyme extraction: Natural bacterioplankton communities were collected from the coastal North Sea during the spring bloom of 1999 using acid-rinsed carboys. Seawater samples (50 liters) were filtered through 0.8-μm pore size polycarbonate filters (142-mm diameter, Millipore) in order to exclude almost all eukaryotic organisms. To minimize the loss of bacterial biomass due to clogging, the filter was replaced every 10 liters. Bacteria in the filtrate were concentrated to a final volume of 0.5 liters using a Pellicon (Millipore) tangential flow filtration system equipped with a 0.1-μm pore size filter cartridge (Durapore, Millipore). Bacteria in the retentate were further concentrated by centrifugation (20,000 *x g*; 30 min; 4°C). The resulting pellet was washed three times with artificial seawater and finally resuspended in extraction buffer consisting of 40% glycerol (Sigma), 100 mM taurine (Fluka), 20 mM cholic acid (Fluka), 1 mM $MgSO_4$, pH 7.50. Bacterial ectoenzymes were extracted by sonication on ice in an ultrasound bath at 50 W for 30 s and centrifuged again (20,000 *x g*; 60 min; 4°C). Thereafter, the supernatant containing the enzyme extract was carefully siphoned off and stored at 4°C for subsequent analysis. The extraction efficiency of β-glucosidases from natural bacterioplankton communities using the above method was, on average, 73.4 ± 9.7% ($n = 5$) as determined by bulk measurements of β-glucosidase activity using the substrate analog methyl-umbelliferyl-β-D-glucoside (described below).

Verification of the ectoenzymatic nature of the measured activity: The bulk of the intracellular components such as DNA and RNA remained in the pellet as verified by phenol-chloroform nucleic acid extraction and agarose gel electrophoresis. An additional experiment was performed in order to determine whether the extracted ectoenzymes measured were free of contamination with intracellular enzymes. Four marine bacterial isolates (*Vibrio sp. Salinicoccus roseus, Paracoccus alkenifer,* and *Planoccocus citreus*) were cultured in ZoBell 2216 broth (5 g of peptone, 1 g of yeast extract, 1 liter of 0.2-μm pore size filtered seawater) amended with 0.5 g of glucose. One milliliter aliquots of exponentially growing cultures were harvested by centrifugation (3,200 *g*; 15 min; 4°C) and washed three times with 0.2-μm pore size filtered seawater. Triplicate aliquots of the bacterial pellets were incubated at 37°C for 30 min in 0.5 ml of a buffer containing 10 mM Tris, 1 mM EDTA, 1 mg lysozyme. Thereafter, Triton X-100 was added (0.1% w/v final concentration) and the cells were sonicated on ice in an ultrasound bath for 30 s and centrifuged (20,000 *x g*; 60 min; 4°C). The purpose of this treatment was to extract as much as possible of the intracellular enzyme pool, and the supernatant was used as a control for total intracellular enzyme activity. At the same time, replicate aliquots of the bacterial pellets were treated as described above for the extraction of ectoenzymes with 0.5 ml of the ectoenzyme extraction buffer.

We measured glucose-6-phosphate dehydrogenase (G6PDH) as a cytoplasmic marker (Martinez and Azam 1992) in the lysozyme-treated extracts, the corresponding ectoenzyme extracts, and in the bacterial pellets after ectoenzyme extraction. The extracts (0.5 ml) or the remaining pellet were diluted to a final volume of 4 ml in a buffer containing 50 mM Tris, 3.3 mM glucose-6 phosphate, 100 μM nicotinamide-adenine dinucleotide phosphate (NADP), 3.3 mM $MgCl_2$, and 1 mM dithiothreitol. G6PDH activity was determined as the rate of NADP reduction by measuring the increase in absorbance at 340 nm in a spectrophotometer.

Bulk measurements of enzyme activity: The bulk β-glucosidase activity of the freshly collected bacterial communities and the extraction efficiency for β-glucosidase were assessed using the fluorogenic substrate analog methyl-umbelliferyl-β-D-glucoside (Hoppe 1983). Fluorescence was measured at an excitation wavelength of 360 nm and an emission wavelength of 445 nm in a Hitachi F-2000 spectrofluorometer.

Capillary electrophoretic (CE) separation and online detection of β-glucosidase activity: A high sensitivity isoenzyme fingerprinting method (Xue and Yeung 1994) was modified and optimized for the detection of the number and relative amount of the different β-glucosidases present. The solubilized enzymes were hydrodynamically injected into the capillary at a constant pressure $\times$ time value of 15 psi $\times$ s to provide a constant injection volume of 147 nl. Thereafter, a voltage of 20 kV was applied for 15 min to separate the different β-glucosidases based on their different electrophoretic mobility and to allow them to migrate into the region containing the substrate. Then the voltage was turned off to allow the enzymes to hydrolyze the fluorogenic substrate analog resorufin-β-D-glucopyranoside (Rglu, Sigma) added to the electrophoresis buffer (composition described below). Hydrolysis of this fluorogenic substrate leads to a local accumulation of the fluorescent hydrolysis product (resorufin) in those regions of the capillary where β-glucosidases are present. After allowing the different β-glucosidases to react with Rglu for 10 min, a 20 kV voltage was applied again to elute the fluorescent products passing the detection window where quantification took place. Migration times were normalized to the average migration time of resorufin standards freshly prepared each day. Only those peaks for which an increase in relative fluorescence in repeated 10- and 20-min incubations was observed were considered as β-glucosidases for further analyses. The fluorescence pattern obtained in this way measures both the richness (number of peaks) and the evenness (peak areas) of the β-glucosidases present in the sample. Repeated sample injections using different concentrations of Rglu (0.1, 0.5, 1, 5, 10, 25, 50, 75, 100, 150, and 200 μM) allowed us to calculate the different kinetic parameters of each separated enzyme. At Rglu concentrations higher than 200 μM, Rglu precipitates in the buffer solution used in this study at 4°C. The kinetic parameters were estimated by nonlinear regression of the peak areas using SYSTAT 9.

Analyses of β-glucosidase diversity were performed using a Biofocus 3000 capillary electrophoresis system equipped with a Biofocus LIF2 laser-induced fluorescence detector (Bio-Rad) as described elsewhere (Arrieta and Herndl 2001). Briefly, the enzyme separation and detection were performed in 50 μm I.D. fused silica capillaries. To avoid electro-osmotic flow and to minimize protein interaction with the wall of the capillary, the inner surface of the capillary was coated with polyacrylamide by means of a siloxane bond (Li 1996). The total length of the capillary was 75 cm, and the distance from the inlet to the detection window was 70.4 cm. The fluorophore (resorufin) released upon hydrolysis of Rglu was measured using the 594 nm line of the helium-neon laser as excitation source; fluorescence emission was measured at 630 nm in the LIF2 system (600DRLP02 beam splitter, 630DF30 discrimination filter, Bio-Rad).

The electrophoresis buffer contained 100 mM taurine, 20 mM cholic acid, and 1 mM $MgSO_4$ dissolved in distilled water, and the pH was adjusted with NaOH to 7.50. The samples containing the solubilized ectoenzymes and buffer solutions were transferred to the refrigerated carrousel of the Biofocus system and kept at 4°C. The capillary temperature was set at 15°C to protect the enzymes from Joule heating during the electrophoretic separation using FC-77 (3M) as cooling fluid.

16S rDNA and 16S rRNA fingerprinting of bacterial communities: Terminal-restriction fragment length polymorphism (T-RFLP) analysis of bacterial communities was performed using primers and methods previously described (Moeseneder et al. 2001), but only one restriction enzyme (*Hha I*) was used in this study.

Results and discussion—We followed the dynamics of the bacterioplankton community during the development and decay of a phytoplankton bloom in the coastal North Sea. A

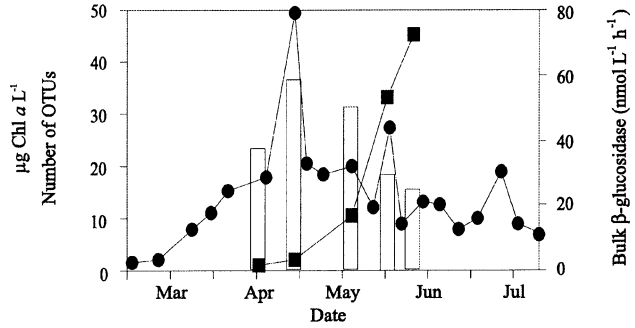

Fig. 1. Temporal dynamics of chlorophyll *a* (circles), bulk β-glucosidase activity (squares), and the number of operational taxonomic units (OTUs) (bars) during the phytoplankton bloom in the coastal North Sea in the spring of 1999.

Phaeocystis-dominated bloom developed in the middle of March, reaching its maximum at the beginning of May and collapsing rapidly thereafter, followed by a smaller diatom-dominated bloom at the beginning of June (Fig. 1). *Phaeocystis* cells not only produce intracellular storage carbohydrates but also large quantities of extracellular mucopolysaccharides that can comprise up to 90% of the colony C-biomass (Rousseau et al. 1990; Janse et al. 1996). As expected, bulk β-glucosidase activity (Hoppe 1983) (Fig. 1) increased exponentially during the collapse of the *Phaeocystis* bloom. This increase cannot be explained by the increase in bacterial abundance alone, as the β-glucosidase activity per cell increased by about 2 orders of magnitude from the beginning to the end of the bloom (data not shown).

Using the CE approach to determine the diversity of β-glucosidase (described above), we observed a total of 11 distinct peaks of β-glucosidase activity over the study period (Fig. 2). At each sampling date, six to eight different β-glucosidases were detected. Thus, the diversity of β-glucosidases in marine systems as estimated by CE is much higher than previously reported (Rath and Herndl 1994), probably due to the superior sensitivity of the CE laser-induced fluorescence (LIF) approach used in this study. Additionally, our CE-LIF procedure is faster (a sample can be analyzed within 24 h of collection) and simpler than traditional enzyme purification approaches.

Glucose-6-phosphate dehydrogenase (EC 1.1.1.49) was determined in four strains of cultured marine bacteria in order to test the possible release of intracellular enzymes during the extraction procedure. The NADP reduction rate measured in the supernatant of the ectoenzyme extracts did not differ significantly from 0 (F-test $P < 0.05$). Even in the remaining pellet, the measured G6PDH accounted for less

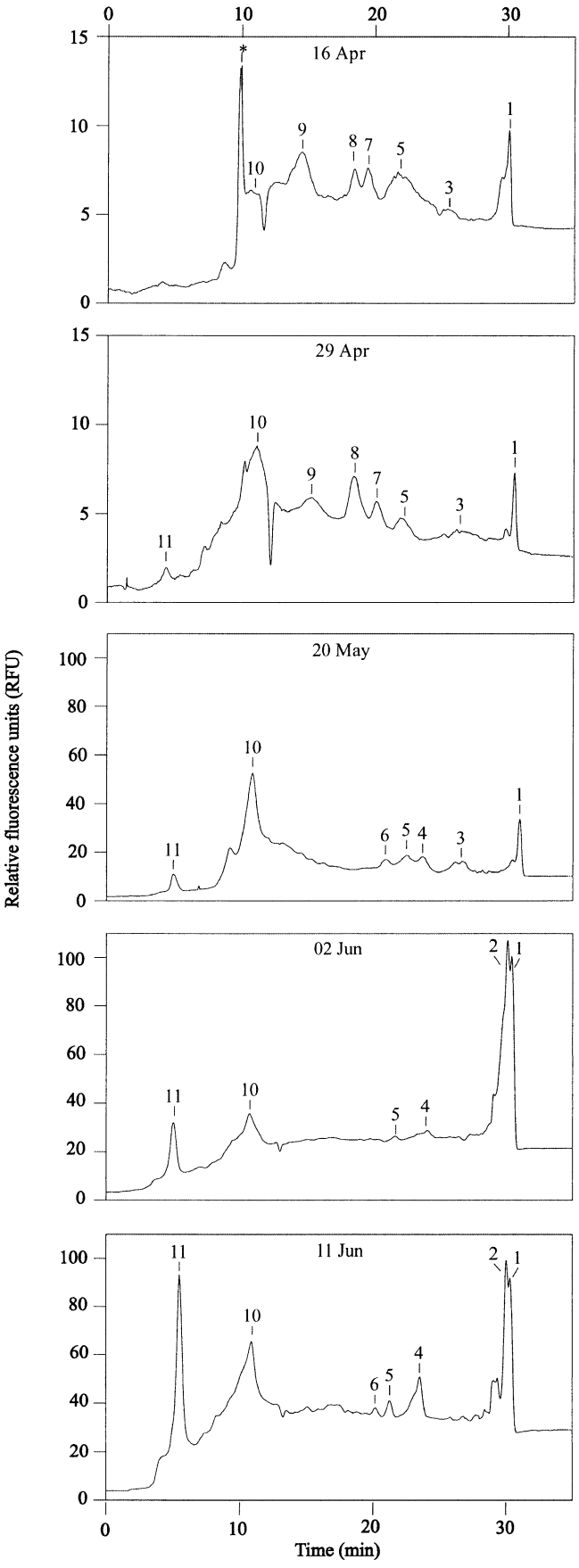

Fig. 2. Profiles of β-glucosidase activity obtained at different stages of the phytoplankton bloom using 50 μM resorufin-β-D-glucopyranoside as substrate for β-glucosidase. Peak numbers correspond to those given in Table 1. Note the different scale of the lower three panels due to the higher β-glucosidase activity. *Not a product of enzyme activity.

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Table 1. Kinetic parameters, V_{max} (nmol L^{-1} h^{-1}) and K_m (μmol L^{-1}), of the different β-glucosidases as estimated by nonlinear regression of the peak area. The values marked with an asterisk are not reliably estimated because the concentrations of substrate used were not high enough to reach saturation level.

β-glucosidase	16 Apr V_{max}/K_m	29 Apr V_{max}/K_m	20 May V_{max}/K_m	02 Jun V_{max}/K_m	11 Jun V_{max}/K_m
Peak 1	0.14/34.6	0.16/32.4	0.67/32.1	11.4/31.5	7.19/34.5
Peak 2	absent	absent	absent	19.14/36.9	14.32/32.4
Peak 3	0.06/37.7	0.11/12.1	0.61/35.8	absent	absent
Peak 4	absent	absent	3.01/17.9	1.2/15.7	4.77/36.1
Peak 5	0.5/136.7*	0.16/269.4*	3.77/282.7*	0.36/234.5*	2.85/242.1*
Peak 6	absent	absent	0.42/114.2*	absent	0.26/18.9
Peak 7	0.15/26	0.15/45.6	absent	absent	absent
Peak 8	0.1/79.8	0.43/88.6	absent	absent	absent
Peak 9	0.19/36.9	0.19/47.6	absent	absent	absent
Peak 10	0.85/70.6	1.71/58.4	7.11/72.3	1.94/97.6	11.78/73.2
Peak 11	absent	0.1/44.5	0.55/34.3	4.39/42.7	18.63/38.1

than 5% of the activity measured in the lysozyme-treated samples in all cases and remained associated to the particulate fraction as determined by centrifugation after the enzyme assay. Therefore, we conclude that the enzymes we measured in our capillary electrophoresis assay were ectoenzymes.

The hydrolysis rates of artificial substrate analogs can differ from those of the naturally occurring substrates, leading to a potential bias in the estimation of the actual hydrolysis rates. The β-glucosidase diversity might be underestimated, as some of the β-glucosidases might not interact with the artificial substrate. In spite of these limitations, our present knowledge of the hydrolytic activities of marine bacterioplankton is mainly based on the use of these analogs, and the information conveyed by such studies has proven useful when these potential limitations are taken into account.

For the detected β-glucosidases, no particular trend was observable in the evolution of the half saturation constant (K_m) values. The K_m values ranged from 26 to 137 μM at the beginning of the study period and spanned a somewhat larger range (12 to 282 μM) after the collapse of the bloom (Table 1). The coexistence of these β-glucosidases with K_m values ranging over one order of magnitude might explain previous findings of multiphasic enzyme kinetics reported for marine systems (Talbot and Bianchi 1997; Tholosan et al. 1999; Unanue et al. 1999).

Additionally, bacterial species richness, i.e., the number of phylotypes, was estimated by T-RFLP analysis of PCR amplified 16S rDNA (Liu et al. 1997; Moeseneder et al. 1999) and reverse transcribed 16S rRNA as a putative indicator for metabolically active bacteria (Poulsen et al. 1993; Moeseneder et al. 2001). From a total of 45 different operational taxonomic units (OTUs) detected at the DNA level, only seven OTUs were present over the entire study period. Bacterial species richness reached a maximum of 36 OTUs at the peak of the bloom and declined to 15 OTUs at the end of the study (Fig. 3c). Decreased bacterial diversity after a phytoplankton bloom has also been observed by others (Pinhassi and Hagström 2000) and is probably a common phenomenon at high resource supply rates (Kassen et al. 2000). The number of OTUs detectable at the RNA level, however, increased after the bloom collapsed. From the OTUs detected at the DNA level, only 31% were detectable also at the RNA level at the peak of the bloom, increasing to 60% in the postbloom community. At the DNA level, the numerical abundance of the organisms is decisive for detection, while on the RNA level, the metabolic activity determines the detection threshold (Moeseneder et al. 2001). Thus, our data suggest that the postbloom bacterioplankton community was dominated by fewer but more active phylotypes as compared to the peak of the phytoplankton bloom.

To test the strength of the relationship between bacterial species richness and β-glucosidase type richness, we computed similarity coefficients for each pair of samples using the observed OTU distribution pattern for both the DNA (species present) and the RNA level (highly active species). We used the simple matching similarity coefficient since our samples are closely related, and therefore the absence of a given character in two samples does convey useful information about the succession. Using a Jaccard coefficient (absent characters ignored) produced the same clustering patterns (data not shown). The same calculation was also performed for β-glucosidase richness. The obtained similarity indexes for β-glucosidase richness significantly correlated with those found for the OTU distribution for both the DNA (Spearman ranked correlation $p = 0.0384$) and the RNA level (Spearman ranked correlation $p = 0.0350$). The dendrogram branching pattern obtained with the rRNA data resembled more closely the pattern obtained for β-glucosidase than on the DNA level (Fig. 3a,b). Thus, the distribution pattern of both β-glucosidase types and OTUs follows a temporal succession (Fig. 3c) and the occurrence of some OTUs is linked to the occurrence of the different β-glucosidase types. These correlations would not be obtained if substrate-induced expression of enzyme activity would trigger the succession of β-glucosidases. Also, in previous tests with marine bacterial isolates, we found only one type of β-glucosidase per bacterial strain (Arrieta, unpubl. data). Even if some bacterial phylotypes express more than one type of β-glucosidase, the number of different β-glucosidases de-

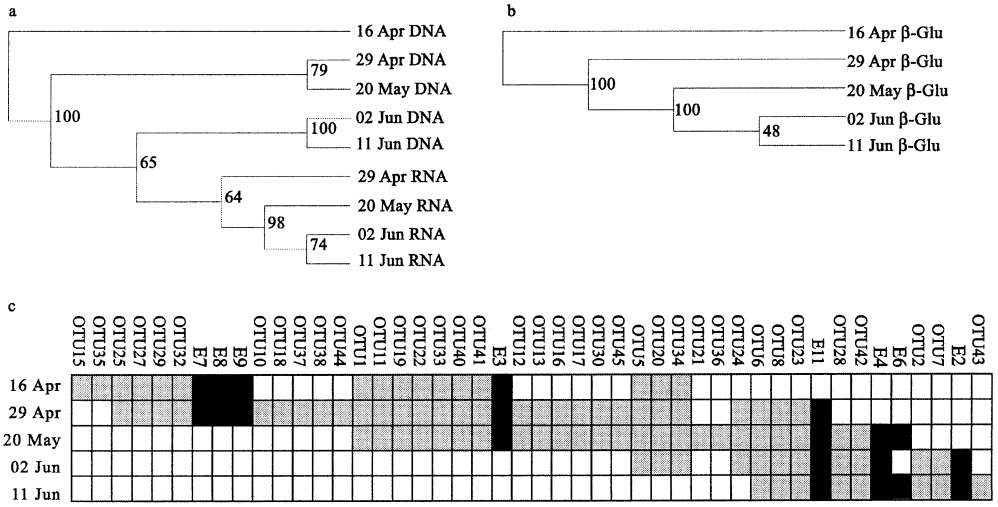

Fig. 3. Dendrograms showing the simple matching similarity between the phylogenetic composition of the bacterial community collected at the different dates and (a) analyzed on the rDNA and rRNA level using T-RFLP and (b) for the β-glucosidase patterns obtained by capillary electrophoresis. Numbers in (a) and (b) indicate the significance of each fork as percentage of occurrence in jackknife resampling analysis. The lower panel (c) shows the temporal succession of OTUs (gray) and β-glucosidases (black) denoted at the *x*-axis by E. The seven OTUs and three β-glucosidases detected in all the samples are not included here.

tected in a sample should be proportional to the number of different β-glucosidase producers present. β-glucosidase richness declined only by 12 to 25% from the maximum observed at the chlorophyll peak toward the end of the study period, while bacterial species richness declined by almost 60% (36 to 15 OTUs) over the same period (Fig. 3c). The even better correspondence with the OTU pattern on the rRNA level clearly indicates that β-glucosidase producers have a selective advantage under postbloom conditions. Our results agree with previous observations (Riemann et al. 2000), suggesting that shifts in bacterial community structure and enzyme activity during the breakdown of the bloom are favored by the availability of new niches and substrates. Thus, we may conclude that the observed increase in β-glucosidase activity was caused by changes in the bacterial community structure rather than by phenotypic plasticity of individual phylotypes.

Although the regulation of enzyme expression by bacteria is a well-known mechanism controlling the hydrolytic activities of bacteria, the data presented here provide the base for an alternative, mechanistic explanation for the regulation of hydrolytic activity in marine bacterial communities. The increase in substrate availability induces changes in the bacterial species composition, favoring the growth of β-glucosidase producers and, therefore, causing an increase in the hydrolysis rate at the community level. These results illustrate the importance of studying functional diversity to mechanistically understand biogeochemical fluxes, since shifts in the dominance of different bacterial populations could strongly affect the rates and patterns of bacterioplankton-mediated hydrolysis of both dissolved and particulate organic matter in the global ocean.

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References

AMON, R. M. W., AND R. BENNER. 1994. Rapid cycling of high-molecular-weight dissolved organic matter in the ocean. Nature **369:** 549–552.

———, AND ———. 1996. Bacterial utilization of different size classes of dissolved organic matter. Limnol. Oceanogr. **41:** 41–51.

ARRIETA, J. M., AND G. J. HERNDL. 2001. Assessing the diversity of marine bacterial β-glucosidases by capillary electrophoresis zymography. Appl. Environ. Microbiol. **67:** 4896–4900.

AZAM, F. 1998. Microbial control of oceanic carbon flux—the plot thickens. Science **280:** 694–696.

CHRÓST, R. J. 1991. Environmental control of the synthesis and activity of aquatic microbial ectoenzymes, p. 29–59. *In* R. J. Chróst [ed.], Microbial enzymes in aquatic environments. Springer.

COTTRELL, M. T., J. A. MOORE, AND D. L. KIRCHMAN. 1999. Chi-

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tinases from uncultured marine microorganisms. Appl. Environ. Microbiol. **65:** 2553–2557.

———, D. N. WOOD, L. Y. YU, AND D. L. KIRCHMAN. 2000. Selected chitinase genes in cultured and uncultured marine bacteria in the α- and γ-subclasses of the proteobacteria. Appl. Environ. Microbiol. **66:** 1195–1201.

GIOVANNONI, S., AND M. RAPPÉ. 2000. Evolution, diversity, and molecular ecology of marine prokaryotes, p. 47–84. *In* D. L. Kirchman [ed.], Microbial ecology of the oceans. Wiley.

HERNDL, G. J., J. M. ARRIETA, AND K. STODEREGGER. 1999. Interaction between specific hydrological and microbial activity leading to extensive mucilage formation in the northern Adriatic Sea. Ann. Ist. Sper. Sanit. **35:** 405–409.

HOPPE, H. G. 1983. Significance of exoenzymatic activities in the ecology of brackish water: Measurements by means of methylumbelliferyl-substrates. Mar. Ecol. Prog. Ser. **11:** 299–308.

———, H. DUCKLOW, AND B. KARRASCH. 1990. Evidence for dependency of bacterial growth on enzymatic hydrolysis of particulate organic matter in the mesopelagic ocean. Mar. Ecol. Prog. Ser. **93:** 277–283.

JANSE, I., AND OTHERS. 1996. Carbohydrates in the North Sea during spring blooms of *Phaeocystis*—a specific fingerprint. Aquat. Microb. Ecol. **10:** 97–103.

KASSEN, R., A. BUCKLING, G. BELL, AND P. B. RAINEY. 2000. Diversity peaks at intermediate productivity in a laboratory microcosm. Nature **406:** 508–512.

LI, S. F. Y. 1996. Chapter 4: Column technology, p. 155–200. *In* Capillary electrophoresis: Principles, practices and applications. Elsevier.

LIU, W. T., T. L. MARSH, H. CHENG, AND L. J. FORNEY. 1997. Characterization of microbial diversity by determining terminal restriction fragment length polymorphisms of genes encoding 16s rRNA. Appl. Environ. Microbiol. **63:** 4516–4522.

MARTINEZ, J., AND F. AZAM. 1992. Periplasmic aminopeptidase and alkaline phosphatase activities in a marine bacterium: Implications for substrate processing in the sea. Mar. Ecol. Prog. Ser. **92:** 89–97.

———, D. C. SMITH, G. F. STEWARD, AND F. AZAM. 1996. Variability in ectohydrolytic enzyme activities of pelagic marine bacteria and its significance for substrate processing in the sea. Aquat. Microb. Ecol. **10:** 223–230.

MIDDELBOE, M., M. SONDERGAARD, Y. LETARTE, AND N. H. BORCH. 1995. Attached and free-living bacteria—production and polymer hydrolysis during a diatom bloom. Microb. Ecol. **29:** 231–248.

MOESENEDER, M. M., AND OTHERS. 1999. Optimization of terminal-restriction fragment length polymorphism analysis for complex marine bacterioplankton communities and comparison with denaturing gradient gel electrophoresis. Appl. Environ. Microbiol. **65:** 3518–3525.

———, C. WINTER, AND G. J. HERNDL. 2001. Horizontal and vertical complexity of attached and free-living bacteria of the eastern Mediterranean Sea, determined by 16s rDNA and 16s rRNA fingerprints. Limnol. Oceanogr. **46:** 95–107.

PAYNE, J. W. 1980. Transport and utilization of peptides by bacteria, p. 212–256. *In* J. W. Payne [ed.], Microorganisms and nitrogen sources. Wiley.

PINHASSI, J., AND Å. HAGSTRÖM. 2000. Seasonal succession in marine bacterioplankton. Aquat. Microb. Ecol. **21:** 245–256.

POULSEN, L. K., G. BALLARD, AND D. A. STAHL. 1993. Use of rRNA fluorescence in situ hybridization for measuring the activity of single cells in young and established biofilms. Appl. Environ. Microbiol. **59:** 1354–1360.

RATH, J., AND G. J. HERNDL. 1994. Characteristics and diversity of β-D-glucosidase (EC 3.2.1.31) activity in marine snow. Appl. Environ. Microbiol. **60:** 807–813.

RIEMANN, L., G. F. STEWARD, AND F. AZAM. 2000. Dynamics of bacterial community composition and activity during a mesocosm diatom bloom. Appl. Environ. Microbiol. **66:** 578–587.

ROUSSEAU, V., S. MATHOT, AND C. LANCELOT. 1990. Calculating carbon biomass of *Phaeocystis* sp. from microscopic observations. Mar. Biol. **107:** 305–314.

SMITH, D. C., M. SIMON, A. L. ALLDREDGE, AND F. AZAM. 1992. Intense hydrolytic enzyme activity on marine aggregates and implications for rapid particle dissolution. Nature **359:** 139–141.

———, G. F. STEWARD, R. A. LONG, AND F. AZAM. 1995. Bacterial mediation of carbon fluxes during a diatom bloom in a mesocosm. Deep-Sea Res. II **42:** 75–97.

TALBOT, V., AND M. BIANCHI. 1997. Bacterial proteolytic activity in sediments of the subantarctic Indian Ocean sector. Deep-Sea Res. II **44:** 1069–1084.

THOLOSAN, O., AND OTHERS. 1999. Biphasic extracellular proteolytic enzyme activity in benthic water and sediment in the northwestern Mediterranean Sea. Appl. Environ. Microbiol. **65:** 1619–1626.

UNANUE, M., AND OTHERS. 1999. Ectoenzymatic activity and uptake of monomers in marine bacterioplankton described by a biphasic kinetic model. Microb. Ecol. **37:** 36–48.

XUE, Q., AND E. S. YEUNG. 1994. Variability of intracellular lactate dehydrogenase isoenzymes in single human erythrocytes. Anal. Chem. **66:** 1175–1178.

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