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Community Dynamics of Free-living and Particle-associated Bacterial Assemblages during a Freshwater Phytoplankton Bloom

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Abstract

Bacterial community dynamics were followed in a 19-day period during an induced diatom bloom in two freshwater mesocosms. The main goal was to compare diversity and succession among free-living (<10 μm) and particle-associated (>10 μm) bacteria. Denaturing gradient gel electrophoresis (DGGE) of PCR amplified 16S rDNA showed the highest number of bands among free-living bacteria, but with a significant phylogenetic overlap in the two size fractions indicating that free-living bacteria were also important members of the particle-associated bacterial assemblage. Whereas the number of bands in the free-living fraction decreased during the course of the bloom, several phylotypes unique to particles appeared towards the end of the experiment. Besides the primer set targeting Bacteria, a primer set targeting most members of the *Cytophaga–Flavobacterium* (CF)-cluster of the Cytophaga–Flavobacterium–Bacteroides group and a primer set mainly targeting α-Proteobacteria were applied. PCR–DGGE analyses revealed that a number of phylotypes targeted by those primer sets were found solely on particles. Almost all sequenced bands from the bacterial DGGE gel were related to phylogenetic groups commonly found in freshwater: α-Proteobacteria, CF, and Firmicutes. Despite the use of primers intended to be specific mainly for α-Proteobacteria most bands sequenced from the α-proteobacterial DGGE gel formed a cluster within the *Verrucomicrobiales* subdivision of the *Verrucomicrobia* division and were not related to α-Proteobacteria. Bands sequenced from the CF DGGE gel were related to members of the CF cluster. From the present study, we suggest that free-living and particle-associated bacterial communities should not be perceived as separate entities, but rather as interacting assemblages, where the extent of phylogenetic overlap is dependent on the nature of the particulate matter.

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Introduction

During the course of phytoplankton blooms, bacteria originating from the surrounding water colonize freshly produced particulate matter. However, a number of studies have shown that bacteria attached to particles may be phylogenetically different from free-living bacteria [7, 13, 15, 41]. This indicates that there are selective forces present in particle microenvironments that gradually drives community succession toward a phylogenetic composition that differs from the composition in the surrounding water. Our knowledge of the importance of different phylogenetic groups in the development of a particle-associated bacterial assemblage is limited and time scales and regulation of this community succession are basically unknown.

Many authors have speculated on the idea of "particle-specialist" bacteria. It was originally based on the apparent superior metabolic capabilities of attached bacteria (e.g., [4, 31, 48, 51, 52]). Recently, the use of different molecular methods [2, 13, 43] as well as a modified microautoradiographic method for measuring activity [20] have supported the perception of free-living and particle-associated bacterial assemblages as metabolically and phylogenetically distinct. However, in a recent study, Hollibaugh et al. [27] observed similarities as well as dissimilarities between the composition of free-living and attached bacterial assemblages in samples collected at different locations in San Francisco Bay (California). They suggested the degree of phylogenetic overlap to be dependent on the particle origin. Thus, there seems to be no consensus as to the interplay between free-living and attached bacterial communities. Further, our knowledge of the phylogenetic compositions of these spatially separated assemblages is limited, especially for freshwater environments.

This prompted us to analyze the community composition dynamics of free-living and attached bacterial assemblages during the course of a freshwater phytoplankton bloom. We hypothesized a scenario where the free-living bacterial assemblage serves a "seed-bank" function [39] harboring a great diversity but with phylotypes specialized in macromolecule hydrolysis and particle-associated growth being present in low numbers waiting to encounter suitable particulate matter. During the course of the phytoplankton bloom, community composition profiles of free-living and especially attached bacterial assemblages were expected to reveal the appearance of new "particle-specialist" phylotypes as particulate matter accumulated and became densely colonized by bacteria. To test this proposed scenario, we compared DGGE profiles of PCR-amplified 16S rDNA from free-living (<10 μm) and particle-associated (>10 μm) size-fractions. PCR–DGGE has been applied for community structure analysis in several recent publications [16, 19, 43, 45, 54] and its potential biases and limitations have been thoroughly reviewed [18, 42, and references herein]. Because of the limited sensitivity of this community-based analysis and the hypothesized low concentration of "particle specialists" among free-living bacteria prior to the phytoplankton bloom, we expected the DGGE profiles of the two size fractions to have only a limited overlap when Bacteria-specific primers were used. Further, the diversity estimated as the number of DGGE bands was expected to be high among free-living bacteria and lower among attached bacteria. Phylotypes found exclusively on particles were expected to occur later in the bloom.

Additionally, we applied a primer set targeting most members of the *Cytophaga–Flavobacterium* (CF) cluster of the Cytophaga–Flavobacterium–Bacteroides group and another targeting mainly α-Proteobacteria. Members of these groups are abundant in freshwater and marine systems [21, 23] and indirect evidence from a marine mesocosm experiment suggests that phylotypes specialized in particle colonization and hydrolysis might be affiliated with these two phylogenetic groups [43]. Members of the CF cluster have been shown to be especially important constituents of particle-associated bacterial assemblages [15, 35, 41], possibly because of their widespread gliding motility and their profound ability to degrade various macromolecules [12, 47]. α-Proteobacteria are rapid colonizers of surfaces in coastal environments [14] and they can periodically comprise substantial fractions of bacterial assemblages on freshwater macroaggregates [56], especially during the early stages of colonization [24]. Therefore, by comparing DGGE profiles of bacterial amplicons to those of CF and α-Proteobacteria we sought to determine possible dynamics within these specific groups, which might not be revealed when general bacterial primers are used in the amplification of community DNA.

Materials and Methods

The experiments were conducted in mesotrophic Lake Esrum (Denmark) in the period 7 to 26 April 1999. Here we report on bacterial community dynamics in two 500-l cylindrical mesocosms made of transparent polycarbonate. The two mesocosms, designated "Nutrients" and "Nutrients+C," were filled with 430 l of 100 μm-filtered surface lake water, moored in the lake, and amended with inorganic nutrients (Si [52 μM], PO_4^{2-} [2 μM], NO_3^- [30

Table 1. Primer sequences and positions

Primer[a]	Position[b]	Sequence (′5–3′)	Target group	Reference
*GC341 F	341–358	CCTACGGGAGGCAGCAG	Bacteria	36
907 R	907–927	CCGTCAATTCA/CTTTGAGTTT	Universal	37
CF319a F	319–336	GTACTGAGACACGGACCA	*Cytophaga–Flavobacterium*	35
*GC534 R	517–534	ATTACCGCGGCTGCTGG	Universal	36
ALF1b F	19–35	CTGGCTCAGAA/GCGAACG	α-Proteobacteria[c]	34

[a] F, forward primer; R, reverse primer.
[b] *E. coli* numbers.
[c] Also matches several members of the δ-subclass of Proteobacteria, most *Spirochetes*, some *Planctomyces*, and a few others.
* A 40 bp GC-clamp was attached to the 5′-end (CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGG; [36]).

μM]) or inorganic nutrients (same concentrations) and glucose (150 μM), respectively. Glucose was added to test for effects of an increased pool of labile carbon on the structure and function of the microbial assemblage. Sampling was initiated the day after amendment and was carried out using acid-washed polycarbonate bottles after thorough mixing of the enclosures with a plastic paddle. Temperature increased during the experiment from 5.3 to 8.7°C. Details of the experiment have been reported by Søndergaard et al. [53] and data on chlorophyll *a* (chl *a*) concentrations, adopted from Søndergaard et al. [53], and bacterial abundance (NH Borch, pers. comm.) are outlined to illustrate the course of the phytoplankton bloom.

The samples for bacterial counts were stained with SYBR Green I (Molecular Probes), fixed with glutaraldehyde (1.8% final), and counted with a FACSCalibur flowcytometer (Becton Dickinson) using 2 μm beads (Polysciences Inc.) for volume calibration. Samples for particle-associated bacteria (retained on 10 μm pore-size polycarbonate filters, Poretics) were sonicated to disperse the bacteria from the particles prior to flowcytometer counting. Dispersion was verified microscopically and previous experiments have shown that bacterial counts are unaffected by the sonication procedure [58].

DNA was obtained on days 1, 5, 8, 13, and 19. Within 2 h of sampling, 100 ml was gravity filtered through 47 mm diameter, 10.0 μm pore-size polycarbonate filters (Poretics) followed by a wash with 5 ml of bacteria-free water (<0.2 μm). The filtrate was then filtered through 25 mm diameter, 0.2 μm membrane filters (Supor-200, GelmanSciences). Filters were frozen at −80°C until extraction. For nucleic acid extraction, the 0.2 μm filters (with "free-living" bacteria) were cut in pieces and vortexed in 400 μl TE (10 mM Tris–1 mM EDTA; pH 8.0). Cells were lysed by a freeze–thaw–boiling protocol: 5 min at −80°C, 10 min at 20°C, and 10 min in a heating block (Grant QBT2, Merck Eurolab, Albertslund, Denmark) at 102°C. Cell debris and filter pieces were removed by centrifugation (15,000 × *g*, 10 min). DNA in the supernatant was precipitated with ¼ vol 10.5 M sodium acetate and 3 vol 96% ethanol, washed with 70% ethanol, and resuspended in 50 μl TE. One-half of the 10.0 μm filters (with "attached" bacteria) were vortexed in 110 μl TE and freeze–thaw–boiled as above. Additional purification steps were necessary because of the high loads of organic matter on these filters. After boiling, the samples were treated with proteinase K (3.6 mg ml^{-1}) at 37°C for 1 h. Then 600 μl 6 M guanidine thiocyanate in TE buffer was added and incubated for 5 min at 102°C followed by addition of 1.2 ml 6 M NaI and 3 mM 1,4-dithiothreithol (DTT, Roche) in TE-buffer and incubation for 20 min at room temperature. Particulate matter was pelleted and 10 μl QiaexII suspension (Qiagen) was added to the supernatant. To ensure efficient binding of DNA to the QiaexII particles, the tube was gently turned over every 2 min for 10 min to keep the particles in suspension, followed by centrifugation and removal of the supernatant. The QiaexII particles with the DNA were washed twice with a mixture of 2 M guanidine thiocyanate and 4 M NaI and 3 mM DTT in TE buffer followed by three washes with 80% ethanol with centrifugations in between. After airdrying, the QiaexII particles were resuspended in 50 μl TE-buffer and heated at 50°C for 10 min to release DNA from the particles. After centrifugation, the supernatant containing the extracted DNA was stored at 4°C until it was used for PCR. All centrifugations were at 15,000 × *g* for 3 min at 4°C.

Bacterial 16S rDNA was amplified by PCR using forward primers intended to be specific for Bacteria (GC341 F–907 R), the *Cytophaga–Flavobacterium* cluster (CF319a F–GC534 R), and for α-Proteobacteria (ALF1b F–GC534 R) combined with reverse universal primers (Table 1). PCR reactions (100 μl) contained 1 × PCR buffer (Boehringer Mannheim), 2.25 mM $MgCl_2$, 0.8 mM deoxynucleotide triphosphates, 0.5 μM of each primer, 2 U Taq DNA polymerase (Boehringer Mannheim), 200 μg bovine serum albumin (Sigma), and 2–5 ng of DNA. Initial denaturation was at 94°C for 5 min followed by thermal cycling programs as follows. Bacterial primers: Denaturation for 1 min at 94°C; primer annealing for 1 min at an initial 65°C, decreasing 1°C every two cycles to a final of 56°C; primer extension for 3 min at 72°C. Twelve cycles were run at 56°C for a total of 30 cycles followed by a final 10 min incubation at 72°C. α-Proteobacterial primers: Denaturation and extension temperatures as above, but with an initial 58°C annealing temperature, decreasing 1°C every 4 cycles to a final of 51°C. CF primers: Denaturation and extension temperatures as above, but with an initial 63°C annealing temperature, decreasing 1°C every 4 cycles to a final of 56°C. Negative controls, in which the template was replaced by an equivalent volume of sterile water (Milli-Q-purified, autoclaved) were included in each batch of PCR reactions. The correct size and purity of PCR products were verified by agarose electrophoresis.

Duplicate PCR reactions were pooled, purified using the High

Table 2. Bacterial abundance and chl *a* concentrations during the three stages of bloom

Parameters	Nutrients			Nutrients+C		
	Prebloom phase (day 1)	Peak[c] (days 5–11)	Postbloom (day 19)	Prebloom phase (day 1)	Peak[c] (days 5–11)	Postbloom (day 19)
Chl *a* ($\mu g\ l^{-1}$)[a]	36.8	73.6	37.6	34.7	60.5	18.9
Bacteria ($\times 10^6\ ml^{-1}$)[b]						
<10 μm fraction	3.80	4.41	1.47	4.98	6.14	1.66
>10 μm fraction	0.87	5.26	2.82	0.67	5.29	1.70

[a] Chl *a* values are adopted from Søndergaard et al. [53].
[b] Bacterial counts were provided by N.H. Borch (unpublished).
[c] Highest values of the period are shown.

Pure PCR Product Purification Kit (Boehringer Mannheim) or the Amicon Microcon-PCR Centrifugal Filter Devices (Millipore), resuspended in TE buffer, and quantified fluorometrically (PicoGreen, Molecular Probes). Thirty ng of PCR product was loaded on 6% (bacterial and α-proteobacterial primers) or 8% (CF primers) polyacrylamide gels (acrylamide:*N,N′*-methylenebisacrylamide 37:1) containing a denaturant gradient, top to bottom (where 100% is defined as 7 M urea and 40% vol/vol formamide). Electrophoresis was performed with the D Gene System (Bio-Rad) using 1× TAE running buffer (Bio-Rad) at 60°C for 6 h at 150 V (8% gel was run for 4 h). Gels were stained for 30 min in SYBR Gold nucleic acid gel stain (1:10,000 dilution; Molecular Probes), destained for 10 min in 1× TAE, and photographed with UV transillumination using a cooled CCD camera (Hamamatsu, Japan). Dendrograms were constructed from DGGE banding patterns by the software Dendron 3.0 (Solltech Inc., Oakdale, USA) using the Dice coefficient and the UPGMA cluster analysis (unweighted pair-group method using arithmetic average). Since DGGE of PCR amplicons does not give a strictly quantitative view of bacterial community dynamics (e.g., [54], but see [10, 42]) only the presence and position but not the density of bands were employed in similarity analyses.

DGGE bands were excised using a sterile razor blade and DNA eluted overnight at room temperature followed by 2 h at 37°C in 40 μl 1× SSC buffer (0.6 M NaCl, 60 mM trisodium citrate, pH 7). The eluate was centrifuged briefly to pellet acrylamide fragments. DNA in the supernatant was precipitated with 1/10 vol 8 M LiCl and 3 vol 96% ethanol and resuspended in 10 μl TE. Prior to sequencing, the excised bands were reamplified and analyzed on DGGE to confirm migration relative to the original sample. Although only a thin slice of the center of each band was carefully excised, reamplification often yielded several faint bands aligning with bands present in the original sample. This indicated that DGGE bands cannot be assumed pure, but only a significant enrichment of a particular sequence. Therefore to enrich the intended band it was excised from the reamplification pattern, again reamplified, and then rerun on DGGE to confirm identity and purity before being sequenced. The presence of a single band confirmed that the excised band was not a heteroduplex [17, 19]. In some cases when this "purification process" failed to give a single band the reamplified band was cloned using the TOPO TA Cloning kit (Invitrogen). A number of clones were screened to find a clone aligning with the intended band. Then the clone was sequenced. Insert-containing clones were identified by agarose gel electrophoresis of PCR amplicons of the inserts using M13F and M13R primers. DGGE profiles of reamplified, cloned DNA were used to check for heteroduplexes as described previously [42]. Bidirectional sequencing was performed with the ABI Prism Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin Elmer Biosystems) using an automated ABI DNA sequencer (the sequencing facility at GATC GmbH, Konstanz, Germany). Sequences between primer regions were aligned to known sequences using BLAST 2.1 (Basic Local Alignment Search Tool; [3]). Gaps were not taken into account in alignment. All sequences were analyzed by the program CHIMERA_CHECK 2.7 from the Ribosomal Database Project [33]. DGGE band sequences have been deposited in GenBank, using the abbreviations ESR 1 to 26, under the following accession numbers (Bact 1–12, CF 1–9, Alpha 1–5; in order): AF268285–AF268310. ESR refers to Lake Esrum.

Results

Bloom Parameters

Bacterial abundances and chl *a* concentrations in the prebloom phase, at the peak of the bloom, and in the postbloom phase are outlined in Table 2 to illustrate the course of the phytoplankton bloom. The addition of glucose only caused minor differences in microbial abundances and chl *a* concentrations between the two mesocosms. Chl *a* levels increased from an initial level of 35–37 $\mu g\ l^{-1}$ on day 1 to peak values of 55.6 to 73.6 $\mu g\ l^{-1}$ on days 5 to 11. The chl *a* concentration then declined. Substantial colonization of particles was observed as the bloom developed. Particle-associated bacteria (>10 μm fraction) initially had low abundances, below 10^6 cells ml^{-1}, that increased 6 to 8-fold to peak at 5.3×10^6 cells ml^{-1} on day 11. At that time the abundance of particle-associated bacteria was 2 to 6 times higher than that of free-living bacteria (<10 μm fraction).

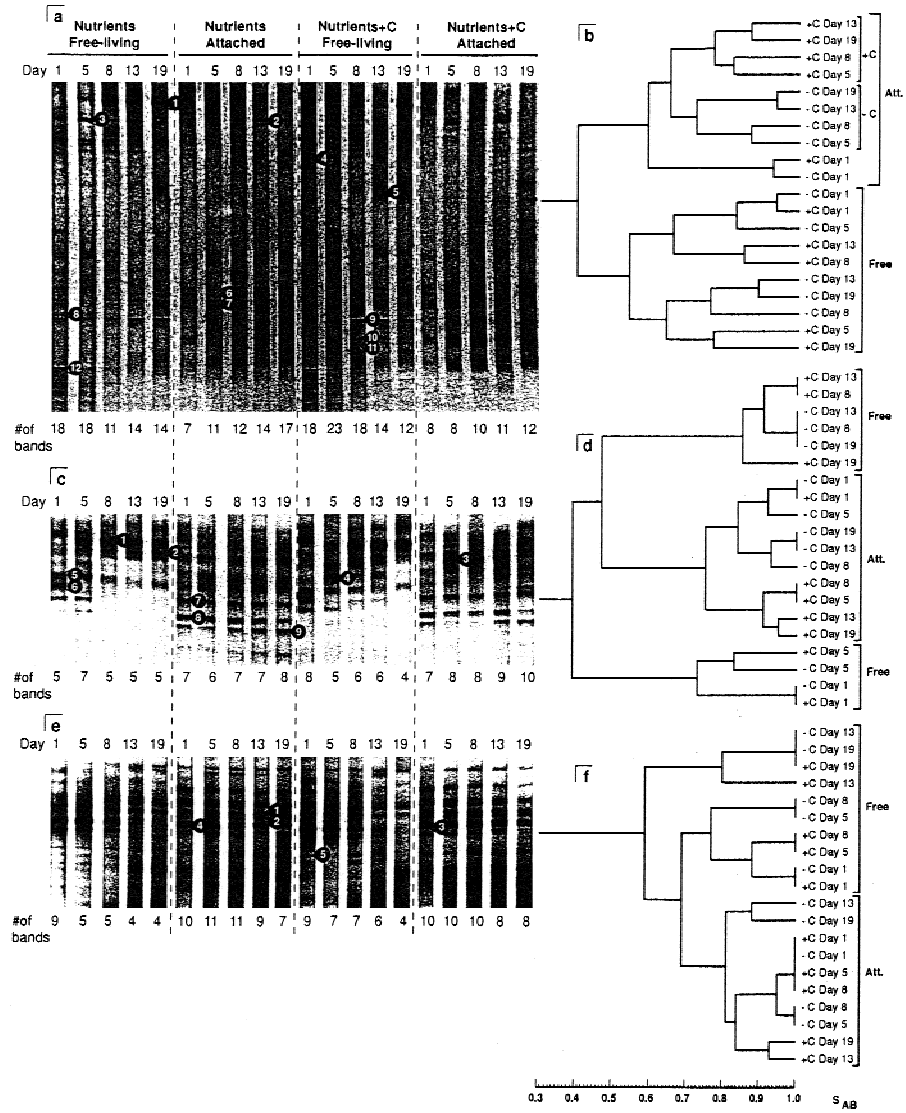

Fig. 1. Bacterial community composition profiles during the course of the phytoplankton bloom analyzed by DGGE of PCR amplified 16S rDNA using primers specific for Bacteria (a), Cytophaga–Flavobacterium (c), and α-Proteobacteria (e). The vertical denaturant gradients applied were 33–53%, 35–54%, and 33–60%, respectively. Excised and sequenced bands are numbered to the right of the lane. The relationships of excised band sequences to other sequences in the GenBank database are indicated in Table 3. Similarity analyses of the DGGE banding patterns are visualized as dendrograms (b, d, and f) to the right of each gel. The number of bands used in similarity analysis is indicated at the at the base of the dendrograms. +C and −C refer to with and without added carbon (glucose).

The abundance of free-living bacteria increased initially, peaked right before the peak of the bloom at 4.4–6.1 × 10^6 cells ml^{-1}, and then declined to a final level of 1.5–1.7 × 10^6 cells ml^{-1}.

The operational distinction between free-living and particle-associated bacteria relied on a 10 μm pore-size filtration, which might have been biased by particles smaller than 10 μm passing the filter and by free-living bacteria being retained on the filter. Flowcytometer counts of the 10 μm filtrate accounted for 89% (SD = 7.6%) of free-living bacteria (defined by their small size as compared to particles) in unfiltered samples, but these counts were statistically similar ($r^2 = 0.97$, $P < 0.001$; NH Borch, pers. comm.). Additionally, it was tested whether the 10 μm filter gradually retained more free-living bacteria as more organic matter accumulated on the filter on day 16. There was no significant difference in counts of the first 10 ml of filtrate as compared to the last 10 ml of the 100 ml filtrate (NH Borch, pers. comm.).

Community Composition Analyses

DGGE profiles of PCR amplified bacterial 16S rDNA fragments are shown in Figs. 1a, 1c, and 1e. In general, profiles from the two mesocosms were very similar in appearance, despite the addition of glucose in one mesocosm and independent of the primer set used. One exception was the bands marked 10 and 11 in the bacterial DGGE gel. These bands appeared in the free-living fraction in nutrients+C, only. Similarity analyses of DGGE profiles are presented as dendrograms (Figs. 1b, 1d, and 1f). Banding patterns changed during the course of the experiment and revealed similarities

Table 3. Closest relatives of the sequenced DGGE bands according to Blast 2.1 (November 2000)

Name	% Similarity	Alignment[a]	Closest relative	Taxonomic description reported in GenBank	Accession no.[b]	References
Bact 1	98.4 %	497/505	Uncultered vent bacterium ML-13	Not reported	AF209008	49
Bact 2	94.0 %	351/536	*Cytophaga/Flexibacter*-like species, clone 70	Cytophagales	AJ225336	11
Bact 3	92.9 %	382/530	Uncultered vent bacterium ML-2d	Not reported	AF208994	49
Bact 4	92.1 %	541/541	Uncultured bacterium, clone BA2	CFB group[c]	AF087043	Unpublished
Bact 5	99.0 %	290/536	Unidentified Cytophagales clone ACK-W3	Cytophagales	U85101	26
Bact 6	98.4 %	548/548	Humic substances enrichment clone A13	β-Proteobacteria	AF231429	Unpublished
Bact 7	98.0 %	548/548	Humic substances enrichment clone A13	β-Proteobacteria	AF173821	Unpublished
Bact 8	95.9 %	391/524	Unidentified bacterium, clone 1404-31	Firmicutes	AJ222835	Unpublished
Bact 9	96.2 %	548/549	β-Proteobacterium F1021	β-Proteobacteria	AF236005	Unpublished
Bact 10	98.3 %	545/549	Humic substances enrichment clone A13	β-Proteobacteria	AF231429	Unpublished
Bact 11	98.3 %	543/545	Humic substances enrichment clone A13	β-Proteobacteria	AF231429	Unpublished
Bact 12	94.0 %	530/530	Unidentified actinomycete, clone ACK-M1	Firmicutes	U85190	26
CF 1	99.4 %	173/176	*Cytophaga* sp.	*Cytophagaceae*	U85890	40
CF 2	100.0 %	176/176	Cytophagales clone CRE - FL24	*Cytophagaceae*	AF141451	13
CF 3	98.9 %	174/176	*Flavobacterium hydatis*	*Flavobacteriaceae*	D12656	38
CF 4	98.9 %	176/176	Soil bacterium SC-I-38	Not reported	AJ252632	Unpublished
CF 5	96.6 %	176/176	*Flavobacterium aquatile*	*Flavobacteriaceae*	M62797	57
CF 6	96.6 %	176/176	*Cytophaga* sp. (type 0092)	Not reported	X85210	9
CF 7	98.9 %	174/176	Soil bacterium SC-I-93	Not reported	AJ252668	Unpublished
CF 8	97.7 %	176/176	*Cytophaga* sp. (type 0092)	Cytophagales	X85210	9
CF 9	96.6 %	174/177	Uncultured bacterium MK18	Cytophagales	AF087072	Unpublished
Alpha 1	93.1 %	479/479	Uncultured clone CRE-PA29	*Verrucomicrobiales*	AF141513	13
Alpha 2	96.7 %	479/479	Uncultured clone CRE-PA29	*Verrucomicrobiales*	AF141513	13
Alpha 3	93.5 %	479/479	Uncultured freshwater bacterium LCK-08	Not reported	AF107318	8
Alpha 4	95.6 %	472/479	Uncultured clone CRE-PA29	*Verrucomicrobiales*	AF141513	13
Alpha 5	99.6 %	458/461	Uncultured clone CRE-FL47	Firmicutes	AF141467	13

[a] Part of the total sequence used in alignment
[b] Nucleotide sequences can be accessed via http://www.ncbi.nlm.nih.gov/Entrez/
[c] Cytophaga–Flavobacterium–Bacteroides group

as well as dissimilarities between free-living and attached bacterial assemblages. To obtain more information on the organisms represented by conspicuous bands on the three DGGE gels, a number of bands were excised from each gel. The excised bands are indicated by numbers (Figs. 1a, 1c, and 1e) and phylogenetic affiliations are reported in Table 3. The 26 sequenced bands showed a high degree of similarity to sequences in the databases (average 96.8%, range 92.1–100% similarity). No heteroduplexes or obvious chimeric sequences were detected among the excised bands. However, it should be noted that from the exclusive use of the CHECK_CHIMERA program of the Ribosomal Database Project, it cannot unambiguously be determined whether a sequence is nonchimeric [33, 44].

Bacterial DGGE

The PCR amplicons generated using Bacteria-specific primers consisted of up to 23 resolvable phylotypes (Fig. 1a). More discernible bands were seen for free-living (11–23) than for attached bacteria (7–17), and whereas the number of bands decreased with time in the free-living fraction, the number increased in the particle-associated fraction. In general, many bands (10 to 12) were unique to the free-living fraction whereas only a few (3 to 4) were unique to particles. Toward the end of the experiment, several bands appeared solely on particles. As expected from visual inspection of the gel, similarity analysis revealed distinct clusters of free-living and particle-associated profiles (Fig. 1b). On day 1, profiles were very similar in the two mesocosms. Whereas the particle-associated fraction in the two mesocosms separated into two distinct clusters later in the experiment, the pattern for the profiles of free-living bacteria was not so clear (Fig. 1b).

Twelve bands were excised and sequenced from the Bacterial DGGE gel (Fig. 1a, Table 3). Five bands (Bact 6, 7, 9, 10, and 11) were closely related to two environmental α-pro-

teobacterial clones and were all closely affiliated with *Aquaspirillum delicatum* (not shown). These five sequences were 94.7 to 99.3% similar (data not shown). Three bands (Bact 2, 4, and 5) were related to environmental clones affiliated with Cytophagales. Bact 2 and 4 were related to clones obtained from activated sludge and Bact 5 was closely related to a clone obtained from the epilimnetic waters of a mountain lake, New York. Bands Bact 8 and 12 were related to Firmicutes clones obtained from Lake Baikal, Russia, and Moss Lake, New York. Bands Bact 1 and 3 were related to sequences obtained from a submarine hydrothermal vent site near Greece.

Cytophaga–Flavobacterium DGGE

The PCR amplicons using CF-specific primers consisted of 4 to 10 resolvable phylotypes in each sample (Fig. 1c). At least 4 distinct bands were unique to attached bacteria whereas at least 2 bands were unique to free-living bacteria. A tendency for more phylotypes to be found on particles (6 to l0 bands) than in the free-living phase (4 to 8 bands) was observed. Similarity analysis revealed that free-living and attached bacteria clustered on different branches and that the profiles changed with time (Fig. 1d). The nine excised and sequenced bands were affiliated with the CF cluster and showed a very high similarity to published sequences (average >98%, Table 3). Two bands (CF 4 and 7) were closely related to bacteria from agricultural soil. Two bands (CF 1 and 2) were related to sequences obtained from coastal environments. Whereas CF 1 was 99.4% similar to an isolate from the Baltic Sea, CF 2 was identical to a clone from the Columbia River estuary, Oregon. Three bands (CF 6, 8, and 9) were related to sequences from activated sludge. Bands CF 3 and 5 were related to *Flavobacterium hydatis* and *Flavobacterium aquatile,* respectively.

α-Proteobacterial DGGE

The PCR amplicons using primers targeting mainly α-Proteobacteria consisted of 4 to 11 resolvable phylotypes in each sample (Fig. 1e). At least 3 bands were unique to the attached bacteria whereas at least 1 band was unique to the free-living bacteria. The diversity of free-living bacteria decreased dramatically with time, which was reflected in the dendrogram where the profiles of the free-living assemblages from the postbloom phase appeared on a distinct branch (Fig. 1f). In general, free-living and attached bacteria clustered on different branches.

For unknown reasons, we had severe problems sequencing bands from the α-proteobacterial DGGE gel. Despite several purification steps and cloning attempts (see Materials and Methods) we were unable to get a satisfying purity for 9 of the 14 excised bands. Only 5 bands yielded a single band at the correct vertical gel position when reamplified and run against the original sample on DGGE. These 5 bands were sequenced successfully; however, surprisingly, no α-proteobacterial sequences were found (Table 3). Four bands (Alpha 1, 2, 4, and 5) were closely related to sequences obtained from the Columbia River estuary, Oregon. Three bands (Alpha 1, 2, and 4) from the >10 μm fractions clustered with the *Verrucomicrobiales* subdivision of the *Verrucomicrobia* division and were closely related (93.1 to 96.7% similarity) to a clone CRE-PA29 obtained from particle-attached bacteria. The 3 sequences were 93.3 to 96.2% similar (data not shown). The Alpha 5 sequence was almost identical (99.6% similarity) to clone CRE-FL47 obtained from free-living bacteria affiliated with Firmicutes (gram-positive bacteria). Band Alpha 4 was related to a clone obtained from an alpine lake and was also closely related to sequences within *Verrucomicrobiales.*

Discussion

Dynamics of Free-Living and Particle-Associated Bacteria

Differences in bacterial community composition were observed between mesocosms with and without added glucose, especially among the attached fractions on the Bacterial profile (Fig. 1b). However, the differences between the fractions (free-living vs attached) were always greater than the differences between treatments. This was observed in spite of the potential biases associated with size fractionation, which might have increased the similarity of DGGE profiles of the two size fractions. Thus, the community composition seemed more influenced by microhabitat than by the availability of glucose.

A surprising finding in this study was that the DGGE analysis of 16S rDNA fragments amplified using Bacteria-specific primers revealed that many bands present in the >10 μm size fraction were also present in the 10 μm filtrate. The dendrogram, based on similarity analyses of DGGE profiles, showed that there was a significant difference in the composition of free-living and particle-associated assemblages;

however, this was caused mainly by the larger number of unique phylotypes in the <10 μm fraction. If it is assumed that conspicuous DGGE bands reflect the presence of prominent, amplifiable phylotypes, then the predominant particle-associated phylotypes were also important members of the free-living bacterial assemblage. Thus, bacteria colonizing particles originated from prominent members of the free-living assemblage and/or there was a substantial release of cells from the particles to the surrounding water. However, in this context, it should be emphasized that the conclusion relies on two important assumptions: (1) that DGGE bands with the same migration distance have identical sequences, which is commonly the case [16, 42], but not always ([32] and see Alpha 3 and 4, Fig. 1e); (2) that the size fractionation used provide a clear-cut distinction between free-living and attached bacteria. Our choice of a 10 μm filter, which is a larger pore-size than commonly used (e.g., 1 μm [43], 3 μm [31], 8 μm [1, 2]) was based on the assumption that using smaller pore-sized filters in the size fractionation would result in a significant retention of "free-living" bacteria on the filter during the extensive phytoplankton bloom. In the DGGE analyses, the unique phylotypes present in the <10 μm fraction must be due to unique phylotypes among free-living bacteria. However, phylotypes present in both fractions may be due to particles (<10 μm) "contaminating" the free living fraction or free-living bacteria being retained on the filters. This could have diminished the differences between the fractions. The main difference between the two size fractions was the additional bands in the free-living fraction. This finding, along with our control count (see Results), indicates that the retention of free-living bacteria on the 10 μm filters did not cause the observed similarity of the two fractions, since then all bands would have been observed in both fractions. However, we cannot dismiss the possibility that particulate matter <10 μm was included in the free-living fraction.

A number of major bands in the free-living fraction disappeared during the course of the bloom indicating changes in the phylotype composition. At the same time, several bands gradually appeared in the >10 μm fraction (Fig. 1a). This was concomitant with the major proliferation of bacteria on particles from day 1 to day 11, where the concentration increased 6–8-fold to 2–6 times the concentration of free-living bacteria on day 11. On the last day of sampling several new phylotypes appeared on particles (see Fig. 1a, Nutrients, Attached, day 19).

In contrast to our findings, a very limited overlap in the phylogenetic compositions of free-living and particle-associated bacteria have been reported for marine snow [15, 41], for aggregates >8 μm in the oligotrophic Mediterranean [1, 2], and for aggregates 3–10 μm from an estuary [13]. We believe this contrast to be a logical consequence of differences in age, and thereby the composition, of the particulate matter investigated. For instance, marine and lake snow is often colonized by microbial assemblages having low production rates and high ectoenzymatic activities [24, 30, 50], which may indicate a highly selective environment that may even be a poor habitat for microbial growth [5, 29]. Thus, older particulate matter would indeed be expected to harbor specialized bacterial communities.

We propose that changes in the phylogenetic composition take place with time after the colonization of freshly formed particulate matter leading to the establishment of specific aggregate-adapted assemblages, as suggested by several recent studies [24, 43, 56]. This could explain the apparent discrepancy between our findings and the prevailing information from other studies, though it should be noted that differences in methodology between this study and studies using cloning/sequencing (e.g., [13, 15, 41]) might be of importance. However, in a recent study of DGGE profiles from San Francisco Bay (California), Hollibaugh et al. [27] observed high similarities as well as dissimilarities between free-living and attached bacterial assemblages at different stations. In accordance with our perception, they suggested that particle origin and composition are important determinants of the similarity of attached versus free communities.

Free-living bacteria can have very high growth potentials [6] and may indeed be physiologically capable of the high cell-specific growth rates measured for cells colonizing freshly produced particulate matter [20, 43, 52]. Heavily colonized particles may function as "baby machines" [28] where attached bacteria release their progeny to the surrounding environment [4, 20, 52]. Thus, in a scenario with freshly formed phytoplankton detritus there may be an extensive exchange of cells between particles and the free-living phase, which will result in a significant overlap in the phylogenetic compositions of the two assemblages, as observed in the present experiment. In our experiment the transport of cells from particles to the free-living phase was not estimated; however, it was presumably significant because of the extremely high concentrations of attached bacteria (Table 2). We therefore suggest that free-living and particle-associated bacterial communities should not be perceived as separate entities, but rather as interacting assemblages, where the ex-

tent of phylogenetic overlap is dependent on the nature of the particulate matter (e.g., origin, age, structure, and composition).

Comparison of Primer Sets

Performing DGGE analyses of PCR amplicons using primer sets for CF or α-Proteobacteria we sought to get a more nuanced view of dynamics from within these two groups. The DGGE profiles were very different from the profiles using general Bacterial primers. As on the Bacterial DGGE gel, bands common to both size fractions or unique to either fraction could be observed. However, the number of bands was significantly lower (≤11 bands) with more bands being discernible in the attached fraction. The difference in the profiles of free-living and attached bacterial assemblages was primarily the result of bands unique to particles and to a rather low diversity among free-living bacteria, which is in marked contrast to profiles of bacterial amplicons. This indicates that a number of the phylotypes targeted by the CF- and α-Proteobacteria primer sets were found solely on particles. Riemann et al. [43] observed a profound dominance of *Cytophaga*-related bands following a diatom bloom in a seawater mesocosm. Similarly, but in a freshwater mesocosm study, Van Hannen et al. [54] observed the appearance of several dominant DGGE bands related to CF 5–6 days after the mass lysis of a pronounced Cyanobacterial bloom. Fandino et al. [16] sampled an intense dinoflagellate bloom off the southern California coast and found that one-third of the sequenced DGGE bands were related to CF. Distinct *Cytophaga*-related phylotypes were found on particles as well as in the free-living assemblage indicating that under conditions of elevated substrate availability members of CF may also become dominant in the free-living assemblage [16]. In freshwater, Weiss et al. [56] and Grossart and Simon [24] found that cells hybridizing to the α-proteobacterial probe, used in our study as a primer, accounted for a significant proportion of the cells on lake aggregates (e.g., up to ca 30% [24]). Thus, our finding of several phylotypes unique for particles on the CF and α-proteobacterial DGGE gels is consistent with reports indicating that some phylotypes matching these primers are specialized in particle colonization. However, it should be noted that a possible dominance of α-proteobacterial or CF-related phylotypes in the bacterial assemblage as particles became colonized could not be confirmed (or thoroughly tested) from the few bands sequenced from the bacterial gel.

Phylogenetic Analyses

The twelve sequenced bands from the bacterial DGGE were related to phylogenetic groups commonly found in freshwater: β-Proteobacteria (5 bands), CF (3 bands), and Firmicutes (2 bands). The closest relatives of Bact 1 and Bact 3 were sequences obtained recently from a hydrothermal vent site; however, these were closely affiliated with phytoplankton plastid genes. The β-proteobacterial phylotypes (Bact 6, 7, 9, 10, and 11) appeared in both size fractions; however, two of the phylotypes (Bact 10 and 11) were found as free-living bacteria only in nutrients+C and not in nutrients. Three phylotypes were related to CF, which is not surprising because of the high abundance of this group in freshwater systems [21]. Notably, two of those (Bact 4 and 5) were only observed in the free-living assemblage. The two bands related to Firmicutes (Bact 8 and 12) were found solely as free-living bacteria and only during the first 5 to 8 days. In contrast, Van Hannen et al. [54] found that Firmicutes phylotypes appeared in the late post-bloom phase of a mesocosm study. Members of the class *Actinobacteria* within Firmicutes have recently been shown to have a broad distribution in freshwater ecosystems [22].

The nine bands sequenced from the CF DGGE gel were related to one another with 90.9–98.9% similarity. This merits its division in different species (see [25]). Interestingly, the nearest relatives of the CF-phylotypes obtained in this study have been found in very different environments, e.g., the Columbia River Estuary, wastewater treatment plants, and agricultural soil, indicating a widespread appearance of CF-phylotypes.

Three of the 5 bands sequenced from the α-proteobacterial DGGE were affiliated with the *Verrucomicrobiales* subdivision of the *Verrucomicrobia* division and a fourth band (Alpha 3) was closely related. This newly described subdivision has been found in a number of different soil and aquatic environments (see [55]) and seems to be widely distributed in lakes [59]. Recently, Zwart et al. [60] showed by DGGE and rDNA sequencing that two sequences within *Verrucomicrobiales* were present throughout the year in a Dutch lake and several *Verrucomicrobiales* isolates have been obtained from German lakes [46]. This suggests that previous studies in lakes, which have applied the so-called "α-proteobacterial" primer used in this study as a probe in *in situ* hybridization (e.g., [24, 39, 56]), may have counted bacteria related to *Verrucomicrobiales* in addition to the intended target of α-Proteobacteria. However, an alignment of the

probe to sequences of cultured members of *Verrucomicrobiales* in GenBank did not yield any 100% match, which might indicate that the target region is not widespread in this group. The fifth band sequenced was related to Firmicutes. No members of Firmicutes had an exact match to the α-proteobacterial primer according to Probe Match 2.1 software (RDP, [33]); however, we found that a number of sequences within Firmicutes had only a single bp mismatch to the α-proteobacterial primer. Nonspecific annealing during PCR cannot be excluded as a cause for the occurrence of these phylotypes in the α-proteobacterial DGGE gel.

The most surprising results of sequencing excised DGGE bands were that (i) sequences from the α-proteobacterial DGGE gel were not related to α-Proteobacteria, and (ii) none of the sequences from the Bacterial DGGE gel related to CF were identical to sequences obtained from the CF DGGE gel. We expected that phylotypes related to CF on the bacterial DGGE gel would be found as bright bands on the CF DGGE gel, since they would presumably be selected by the CF-specific primers and thus would represent an even larger fraction of the amplicons. The specificity of the two primer sets might have caused this variation. Alternatively, the three bacterial DGGE gel bands might not contain the CF signature sequence matching the CF primer (the CF-primer targeted ≈ 90% of the available database CF sequences; [35]), or the lack of sequence overlap between the gels could simply be due to the limited number of bands sequenced.

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