Effect of Nutrient Periodicity on Microbial Community Dynamics

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When microbes are subjected to temporal changes in nutrient availability, growth rate and substrate affinity can contribute to competitive fitness and thereby affect microbial community structure. This hypothesis was tested using planktonic bacterial communities exposed to nutrient additions at 1-, 3-, 7-, or 14-day intervals. Growth rates after nutrient addition were inversely proportional to the pulse interval and declined from 0.5 h⁻¹ to 0.15 h⁻¹ as the pulse interval increased from 1 to 14 days. The dynamics of community structure were monitored by 16S rRNA gene PCR-denaturing gradient gel electrophoresis. At pulse intervals of more than 1 day, the community composition continued to change over 130 days. Although replicate systems exposed to the same pulse interval were physiologically similar, their community compositions could exhibit as much dissimilarity (Dice similarity coefficients of <0.5) as did systems operated at different intervals. Bacteria were cultivated from the systems to determine if the physiological characteristics of individual members were consistent with the measured performance of the systems. The isolates fell into three bacterial divisions, *Bacteroidetes*, *Proteobacteria*, and *Actinobacteria*. In agreement with community results, bacteria isolated from systems pulsed every day with nutrients had higher growth rates and ectoaminopeptidase specific activities than isolates from systems pulsed every 14 days. However, the latter isolates did not survive starvation longer than those provided with nutrients every day. The present study demonstrates the dynamic nature of microbial communities exposed to even simple and regular environmental discontinuities when a substantial pool of species that can catabolize the limiting substrate is present.

In most natural ecosystems microorganisms are likely to experience alternating periods of unrestricted growth with surplus nutrients, nutrient-limited growth, and starvation (6, 35). For example, phototrophs are exposed to light-dark cycles (23), with resulting effects on substrate supply to heterotrophs (1). These nutrient pulses can take place at all scales in nature (36)—over kilometers in coastal marine upwellings (19) and millimeters in laminated microbial mats. For heterotrophic microorganisms, the availability of organic substrates as the carbon and energy source is probably the most significant environmental restriction on growth (24). This is particularly true in unsaturated soil habitats, where nutrient mobility is restricted and surface-attached bacteria may remain dormant for extended periods (6).

The interval between nutrient pulses could have substantial effects on microbial community composition and perhaps on microbial community function. When a nutrient pulse recurs, the physiological traits that are selected may be in opposition to those required for the microbes to survive an extended period of starvation. The exposure of a starved community of bacteria to an energy source results in interspecies competition (9) and was predicted to select for organisms that (i) rapidly regain metabolic competence for growth (27) and (ii) have a high growth rate (24, 40). After the energy source is depleted, the capacity of individual species to survive starvation will be a function of their physiological state (for example, content of nutrient storage polymers) (43), as well as changes in gene expression that lead to a state of reduced metabolic activity (34). In addition, the physiological characteristics of individual

species are overlaid by the capacity of “opportunists” to grow on the organic matter of cells which have not survived starvation in a closed system (3).

These experiments were designed to test the predictions (24) that pulsed environments would select for bacteria that either responded rapidly to nutrient inputs after starvation or survived starvation better than competitors. A system much simpler in nutrient complexity than those found in nature was used in order to also address the functionality of microbial communities. Gelatin (protein) was added as the sole energy substrate; it is typical of the polymeric substrates that are most abundant from decay of biomass in planktonic aquatic systems. Our objective was to determine how substrate pulses affected the physiological activity and the composition of a mixed microbial community.

MATERIALS AND METHODS

Growth of microbial communities. A diverse set of heterotrophic bacteria was inoculated into a set of batch cultures by using activated sludge collected from the aeration tanks of the West Lafayette, Ind., municipal wastewater treatment plant. The activated sludge was first treated with 0.15 mg ml⁻¹ of cycloheximide for 2 h at 30°C in a water bath with shaking to eliminate microeukaryotes. After the incubation, an aliquot was observed under the microscope to verify the absence of microeukaryotes. The treated inoculum was homogenized with a Dounce tissue homogenizer, and 0.5 ml was added to 250-ml Erlenmeyer flasks containing 50 ml of xenobiotic basal medium (XBM) (25) plus 1 g liter⁻¹ gelatin (Difco Laboratories, Detroit, MI). Cultures were incubated in a Gyrotory water bath shaker (New Brunswick Scientific, Edison, NJ) at a speed of about 100 rpm at 30°C.

The microbes were periodically exposed to gelatin (nutrient pulse) by transferring a culture to fresh gelatin medium at a 1:500 dilution. Four different pulse intervals were used (1, 3, 7, or 14 days), and each was run in triplicate for 132 days. Analysis of bacterial growth rate, substrate consumption, cell composition, and specific activity of aminopeptidase was done on days 60 and 90.

Analytical procedures. Bacterial biomass was estimated by measuring the optical density at 600 nm (28) using a Lambda 40 UV/visible spectrometer

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(Perkin Elmer, Norwalk, CT). The growth rate and biomass decay rate were calculated using the slope of $\ln$ optical density at 600 nm versus time by linear regression of the appropriate time intervals. The gelatin concentration in the medium (soluble gelatin) was measured by the method of Lowry et al. (31) after cells were removed by centrifugation at $7,500 \times g$ for 8 min.

To determine the changes in aminopeptidase activity, a fluorogenic substrate analog (7-amino-4-methylcoumarin-L-leucine) (Sigma, St. Louis, MO) was used (15). Ectoaminopeptidase activity was measured as described by Konopka and Zakharova (26). The ATP concentration was determined according to the protocol of Cook et al. (4). RNA was determined by the method of Herbert et al. (12).

Community composition. Changes in community composition were determined by denaturing gradient gel electrophoresis (DGGE) of the PCR-amplified 16S rRNA gene (33). Total genomic DNA was extracted using the Bio 101 Fast DNA kit (QBiogene, Carlsbad, CA), following the manufacturer's instructions. PCR amplification of the 16S rRNA gene was performed using universal bacterial primers PRBA338f (5' AC TCC TAC GGG AGG CAG CAG 3') (with a GC clamp [5'-CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG G-3'] attached to the 5' end) and PRUN518r (5'-ATT ACC GCG GCT GG-3'), which together target the highly variable V3 region (approximately 200 base pairs). DGGE was carried out using the D-Code apparatus (Bio-Rad, Hercules, CA), following the methods described by Morgan et al. (32). Equivalent masses of PCR products were resolved on 8% (wt/vol) polyacrylamide gels (37.5:1 acrylamide:bisacrylamide) with a denaturing gradient of 32.5% to 57.5% (100% denaturant contains 7 M urea, 40% [vol/vol] formamide in 1× Tris-acetate-EDTA). Standards comprised of 16S rRNA gene amplicons from seven phylogenetically different bacterial isolates were included as marker lanes in all gels to enable between-gel comparisons. Representative samples from different gels were also run on the same gel in order to confirm the similarity of their fingerprints.

The DGGE fingerprint profiles were analyzed using Bionumerics software version 2.0 (Applied Maths, Kortrijk, Belgium). Dice similarity coefficients (7) were calculated for all pairwise combinations as two times the number of shared bands divided by the total number of bands found in the pair of samples. To determine the relative differences between samples across time, a nonmetric dimensional scaling (NMDS) analysis was applied to the similarity matrix obtained (SAS System for Windows V8; SAS Institute Inc., Cary, NC). When DGGE data were used for NMDS analysis, the plots represent the relative differences between the compared samples and also changes of a set of samples with time (8). Cluster analysis of samples for a single date was conducted by the unweighted pair group method with arithmetic mean.

DGGE fingerprints are useful tools to characterize and compare microbial communities. However, it is important to recognize their limitations. Overestimates of diversity and/or dominant populations may come from the formation of heteroduplex or chimera molecules during PCR or because of sequence heterogeneities in multiple copies of the 16S rRNA gene. Only a short portion of the 16S rRNA gene (~200 bp) was amplified to minimize chimera formation. Conversely, populations may be underestimated because all sequences are not amplified equally or because gel resolution is poor.

Isolation and characterization of bacteria. Isolates were obtained from each of the 12 systems on day 90. Serial dilutions in XBM were made and dilutions (10^5 to 10^7) were spread onto gelatin plates and incubated at 30°C. Cultures were purified from single colonies by standard microbiological techniques. DGGE was performed as described above for all the isolates to identify unique isolates from a pulse interval. These isolates were selected for phylogenetic and physiological analysis. The near-full-length sequence (1,506 to 1,537 bp) of the 16S rRNA gene was determined as described by Morgan et al. (32). A phylogenetic tree was generated using CLUSTAL X (41) and the neighbor-joining method (37) with 1,000 bootstraps. Included in the analysis were database sequences that best matched our unknowns.

The exponential growth rate was determined for each isolate on XBM plus gelatin (2 g liter⁻¹) as a carbon and energy source, using 96-well microplates agitated at 1,000 rpm at 30°C (Jitterbug microplate shaker; Boeckel Scientific, Feasterville, PA). Increases in optical density at 600 nm were measured using a Versamax microplate reader (Molecular Devices Corporation, Sunnyvale, CA). The growth rate was calculated as indicated above for the mixed communities based on five replicates of each isolate. Growth kinetics were also measured after different lengths of time in stationary phase, to determine if isolates differed in their reactivity after various periods of nutrient absence. Isolates were grown on a shaker at 30°C for 1 day to reach stationary phase. They were then incubated statically at 30°C, and samples were removed to determine growth rate after 1, 3, 7, or 14 days.

The death rate was determined using the method of Hiraoka and Kimbara (13) with some modifications. This method defines dead cells as those permeable to

propidium iodide. Each isolate was inoculated into 25 ml of XBM plus gelatin (2 mg ml⁻¹) and incubated in a Gyrotory water bath shaker (New Brunswick Scientific, Edison, NJ) at a speed of 100 rpm at 30°C until the culture reached stationary phase. Cells were centrifuged and washed with 10 mM Tris-HCl buffer, resuspended in 25 ml of 10 mM Tris-HCl (pH 7.5) buffer, and incubated in a shaker at 30°C. At each time point, two aliquots of 0.5 ml were removed. One was killed with 0.5 ml of 95% ethanol on ice for 15 min (total particulate biomass), and the other was diluted with 0.5 ml of 10 mM Tris-HCl, pH 7.5 (experimental sample). At time zero (early in stationary phase), a killed sample was stored at 4°C and was used as the reference for total microbial biomass at the start of the experiment. Propidium iodide (Sigma Chemical Co., St. Louis, MO) has been used to evaluate membrane integrity because it can penetrate damaged membranes but is excluded by intact cell membranes (13). Propidium iodide was added to both samples to a final concentration of 5 µg ml⁻¹, followed by a 5-min incubation in the dark at room temperature. Dye was also added to a 10 mM Tris-HCl (pH 7.5) blank. Red fluorescence was measured on a TD-700 fluorometer (Turner Designs, Sunnyvale, CA) using a rhodamine filter. For each isolate, the fluorescence yield when all cells were dead was measured by using the ethanol-killed sample. Fluorescence output was then measured for the experimental sample and the blank. The fluorescence units (FU) obtained for the blank were subtracted from those for both the sample and the ethanol-killed sample. The corrected FU for the experimental sample was divided by the corrected FU for the ethanol-killed sample to calculate the fraction of dead cells. The proportion of live cells was calculated by subtracting the percentage of dead cells from 100. The first-order death rate based on the slope of $\ln$ live cells versus time was determined by linear regression of the appropriate time intervals.

Aminopeptidase activity was measured to determine the ability of isolates to utilize the protein substrate and whether enzymes were localized on the cell wall or released into the external medium. Isolates were grown on gelatin medium (2 mg/ml) to mid-logarithmic phase. A volume (1 to 2 ml) of culture was centrifuged at 14,000 rpm for 5 min at room temperature, and the supernatant fraction was analyzed. An aliquot (100 to 500 µl) of uncentrifuged culture was also analyzed. Ectoaminopeptidase activity was determined as described above. The activity of the supernatant fraction was subtracted from the total activity to obtain cell-associated activity.

Nucleotide sequence accession numbers. The 16S rRNA gene sequences reported in this study were deposited in the GenBank database under accession numbers DQ298754 to DQ298787.

RESULTS

Growth kinetics. For each pulse regimen, the three replicate cultures had similar dynamics of growth and decay. The kinetics of substrate consumption and growth slowed as pulse length increased (Fig. 1). Gelatin was consumed after 5, 10, 20, and 30 h in cultures pulsed every 1, 3, 7, or 14 days. The growth rates on day 90 ranged from 0.48 ± 0.01 h⁻¹ for 1-day pulse intervals down to 0.15 ± 0.02 h⁻¹ for 14-day pulse intervals (mean $\pm$ standard error; $n = 3$) (Table 1). Similar growth rates were also found on day 60 (data not shown). After gelatin addition, the bacterial communities grew without a lag phase for pulse intervals of 1, 3, and 7 days. In contrast, cultures pulsed every 14 days exhibited biphasic exponential growth, with an initial slow phase of 0.053 ± 0.01 h⁻¹ followed by the growth rates stated above. After substrate was consumed, turbidity decreased at a first-order rate in cultures pulsed at 3-, 7-, or 14-day intervals. This rate ranged from 0.002 to 0.016 h⁻¹ and was not related to the pulse interval.

The dynamics of several physiological characteristics (cellular activity and cellular composition) were measured over the pulse interval on day 90 (Fig. 2). Ectoaminopeptidase activity increased after substrate addition and declined after gelatin was consumed. Replicate cultures generally had similar activity levels; however, a few exceptions were found and are identifiable in Fig. 2 as points with larger standard errors. Ectoaminopeptidase activity was similar for 1-, 3-, and 7-day pulse intervals but was approximately 5 times lower at the 14-day pulse

FIG. 1. Kinetics of bacterial growth (■) and substrate consumption (●) after gelatin addition to batch cultures pulsed every (A) 1 day, (B) 3 days, (C) 7 days, and (D) 14 days. These kinetics analyses were performed on day 60. Representative data for only one of the three replicate systems at each pulse interval are presented. The inset in panel D details the changes during the first 15 h. OD₆₀₀, optical density at 600 nm.

interval. Maximum ATP levels were about 2 times lower in cultures pulsed every day in comparison to the other pulse regimens, in which the levels were as high as 17 nmol ATP mg cell protein⁻¹ (Fig. 2). RNA levels remained relatively constant at about 60 µg RNA mg cell protein⁻¹ in cultures pulsed at 1-day intervals. The other treatments exhibited dynamics in RNA and ATP content during the course of a substrate pulse, with maximum values occurring during the exponential growth phase. These maxima were two- to threefold higher for RNA and four- to fivefold higher for ATP than the baseline values observed during starvation. Systems that experienced long starvation phases retained RNA contents that were 50 to 70% of those found in cultures that received daily inputs of substrate.

Community dynamics. The community composition was assayed from DGGE fingerprints based upon amplification of 16S rRNA genes, with the intent to analyze changes due to differences in pulse interval. However, the temporal changes in an individual system (Fig. 3) and the differences among replicate systems exposed to the same pulse interval (Fig. 4) were of similar magnitude to the differences between pulse intervals. Multivariate statistical analysis showed that communities in the 1-day pulse interval changed continuously for the first 25 transfers (162.5 generations). Over the subsequent 650 generations the changes were modest, with similarity coefficients ranging from 0.97 to 1 between days 25 and 132, as illustrated by the cluster of data on the NMDS plot (Fig. 3A). This level of stability was never reached by the other pulse intervals, although changes in community composition were also highest during the first transfers (Fig. 3B, C, and D). When multiple samples were taken from a system during one pulse interval (only in 14-day interval systems), relatively small changes in community structure were found (*s* ranged from 0.7 to 0.9) (Fig. 3D).

The number of DGGE bands ranged from 9 to 17 through the length of the experiment for the 1-day pulse interval. The average band number ranged from 10 to 12 for the 3-day pulse interval, from 7 to 15 for the 7-day pulse interval, and from 13 to 14 for the 14-day pulse interval. These averages were similar among the three replicates for 3- and 14-day intervals. Conversely, the replicates of the 1- and 7-day pulse intervals varied twofold in the number of bands.

In order to compare communities that had undergone the longest period of selection, analyses were performed using the last four samplings (days 98, 105, 118, and 132). After 98 days, temporal changes in community structure have diminished, but because community composition was not static, the total number of bands present was determined by pooling data for the 12 fingerprints (three replicates over these last four dates) for each of the four pulse intervals. The number of bands was similar for the 1-, 3-, and 7-day pulse intervals (Table 2) but twofold higher for the 14-day interval. This analysis demonstrated the community heterogeneity found in replicate systems exposed to the same pulse interval. From 37 to 67% of the band classes detected in the systems operating at a specific pulse interval were uniquely found in only one of the three replicates. Only 3 to 18% of the band classes were detected in all three replicate systems at a pulse interval. Cluster analysis of the 48 DGGE fingerprints by the unweighted pair group method with arithmetic mean illustrated that the four samples from an individual system (analyzed on days 98, 105, 118, and

TABLE 1. Growth rates of mixed communities and pools of isolated bacteria from systems exposed to nutrient pulses at 1-, 3-, 7-, or 14-day intervals^a

Pulse interval (days)	Growth rate ^b (h ⁻¹ , mean $\pm$ SE) of community ($n = 3$)	n^c	Growth rate ^d (h ⁻¹ , mean $\pm$ SE) at day after incubation:			
			1	3	7	14
1	0.48 $\pm$ 0.01	9 (6)	0.47 $\pm$ 0.03	0.43 $\pm$ 0.03	0.32 $\pm$ 0.02	0.33 $\pm$ 0.03
3	0.35 $\pm$ 0.04	8 (8)	0.41 $\pm$ 0.03	0.37 $\pm$ 0.03	0.29 $\pm$ 0.02	0.32 $\pm$ 0.03
7	0.29 $\pm$ 0.02	10 (5)	0.48 $\pm$ 0.03	0.45 $\pm$ 0.04	0.37 $\pm$ 0.04	0.34 $\pm$ 0.05
14	0.15 $\pm$ 0.02	5 (3)	0.27 $\pm$ 0.09	0.29 $\pm$ 0.07	0.27 $\pm$ 0.07	0.20 $\pm$ 0.04

^a Bacteria were isolated after systems were operated for 90 days.

^b The community growth rates that are reported were those measured after 90 days of operation.

^c n , number of isolates. The number of isolates in which the mobility of PCR amplicons matched a band in the corresponding mixed community is given in parentheses.

^d Average growth rates for all isolates obtained from a pulse interval, when tested after different intervals since nutrient addition. The values in boldface correspond to the incubation period that corresponds to the pulse interval from which the bacteria were isolated.

132) clustered nearest each other for 9 of the 12 systems, with Dice similarity values of 0.9 to 1 for 1-day pulse intervals and 0.7 to 0.9 for the other pulse intervals. However, the three replicates of a pulse interval did not cluster closely with each other and had Dice similarity coefficients that ranged from 0.4 to 0.6, a range similar to that found when comparing communities exposed to different pulse intervals.

Characterization of bacteria isolated from the cultures. After 90 days of cultivation, representative bacterial strains were isolated from each of the 12 cultures. Strains that had unique DGGE bands for each pulse interval were selected for further physiological analyses. A total of 32 different isolates were obtained from the systems, i.e., 9, 8, 10, and 5 cultures from the 1-, 3-, 7-, and 14-day pulse intervals, respectively. Isolates are coded as the pulse interval from which they were isolated (1, 3, 7, or 14), followed by the replicate (A, B, or C) from which they came. The majority (24 of 32 [75%]) of the isolates have a DGGE band that comigrated with a band found in the mixed community on the day of isolation (Table 3). Sequencing analyses of 16S rRNA genes indicated that all isolates were members of one of three phyla: *Bacteroidetes*, *Proteobacteria* (α , β , or γ), or *Actinobacteria* (Table 3).

We had hypothesized that different pulse intervals would select for organisms with different physiological characteristics. Therefore, the growth kinetics in response to a gelatin pulse were determined for all isolates after 1, 3, 7, or 14 days had lapsed since their last exposure to gelatin (Table 3). The range of maximum growth rates among the groups of isolates from 1-, 3-, or 7-day pulse intervals was relatively small, as indicated by the relatively small coefficients of variation (<30% of the mean) for each of these three groups. The growth rates for the group of isolates obtained from systems pulsed every 14 days were more variable. The coefficients of variation were >40% of the mean, with a range of growth rates from 0.006 to 0.49 h⁻¹. When the growth rates of isolates were evaluated at the same pulse interval as that of the system from which they were isolated and compared to the mixed-community growth rate, the average values were similar (Table 1). The reported average values are for all isolates obtained at that pulse interval, but the same relationship was found if the analysis was restricted to only those isolates for which we found corresponding bands in the PCR-DGGE fingerprints. Analysis of variance indicated that the growth rates of isolates from the 14-day pulse intervals were

FIG. 2. Changes in ectoenzyme activity ($\mu\text{mol MUF/h/mg}$ cell protein) ($\blacktriangle$), ATP ($\text{pmol ATP/mg cell protein}$) ($\bullet$), and RNA ($\mu\text{g RNA/mg cell protein}$) ($\blacksquare$) in batch cultures during a pulse interval on day 90. Gelatin was added at time zero. Cultures were pulsed (A) each day, (B) every 3 days, (C) every 7 days, or (D) every 14 days. The data points give the means for triplicate cultures, and the error bars are the standard errors of the means.

FIG. 3. Nonmetric dimensional scaling plot of the differences in bacterial community structure based on 16S rRNA gene DGGE pattern analysis for cultures subjected to (A) 1-, (B) 3-, (C) 7-, or (D) 14-day intervals of nutrient addition. The numbers on the plots represent the day on which the sample was obtained. In panel A, many samples mapped to the same locus (shown as a closed circle). For the 14-day pulse interval (D), multiple samples which were taken within a single pulse interval are circumscribed within a line.

significantly lower than those of any of the other groups ($P < 0.01$).

Most organisms initiated growth soon after the introduction of nutrients, particularly in comparison to the length of the starvation period (Table 3). The exceptional strains were 3B1 and 3B3, which were the only isolates with no lag at all the different pulse intervals, and isolate 14C13, which had a long lag of >10 h (and a specific growth rate of 0.006 h^{-1}) after all pulse intervals.

Death rates among the isolates were variable; there were no trends related to the pulse interval at which the isolates were selected (Fig. 5A). In particular, the lag time before the onset of exponential death kinetics was highly variable (Fig. 5B).

The nutrient-unrestricted growth rates of specific isolates were not constant; they tended to decrease when cells had a prolonged period of nutrient exhaustion. One-way analysis of variance of these data indicated that the differences in growth rate were statistically significant ($P < 0.05$) for isolates from 1- and 3-day pulse intervals but not for those selected during 7- and 14-day pulse intervals. As a result, there were relatively small differences in nutrient-unrestricted growth rates among isolates from any pulse interval after 14 days of starvation. Fourteen of the strains (group A) had growth rates that were 30% lower after 14 days of starvation than when nutrients were provided within 1 day. In contrast, 10 of 32 strains (group B) showed almost no change in growth rate. Each group was comprised of isolates from all four pulse treatments. However, the mean growth rate after 1 day of nutrient deprivation for

FIG. 4. Comparison of 16S rRNA gene DGGE fingerprint profiles from three replicate systems operated at different pulse intervals after 90 days of operation. The scale bar indicates the percent similarity at the nodes.

group A (0.46 h^{-1}) was significantly greater than that for group B (0.33 h^{-1}) (Student's unpaired t test, $P < 0.01$).

The changes in growth rate observed after extended starvation could have either a physiological or a genetic basis. *Chryseobacterium* strain 1A9 was tested to examine whether the reduction in growth rate observed after extended starvation was genetically stable. The strain grew at a rate of 0.63 h^{-1} when nutrients were provided each day, but after 14 days of starvation the exponential growth rate declined to 0.32 h^{-1} . When cells from that culture were transferred to fresh medium the next day, the exponential growth rate had reverted to 0.62 h^{-1} . Therefore, the reduction in growth rate after extended starvation was not a stable genetic trait and presumably has a physiological or epigenetic basis.

There were six cases in which isolates with 16S rRNA gene similarities of $>99\%$ were obtained from different pulse intervals. This was particularly pronounced for 9 of the 10 *Gamma-proteobacteria* isolates, which formed two clusters of closely related strains whose sequences were very similar to those of *Stenotrophomonas* species (99%). One group (1B3, 3B1, 7B2, 7B9, and 14A9) contained isolates obtained from systems operated at pulse intervals of 1, 3, 7, and 14 days. The second group (3C10, 7B3, 14A3, and 14B1) came from systems pulsed at 3-, 7-, or 14-day intervals. The clusters of *Bacteroidetes* strains were isolated from either the two shortest or the two longest pulse intervals. Many of the physiological characteristics of related strains (Table 3) were similar within the group, although some differences (particularly in exponential-phase growth rate after a 1-day transfer and in the lag before onset of exponential death) were noted. However, examination of these

TABLE 2. Distribution of DGGE band classes among three replicate systems undergoing substrate pulses of different intervals^a

Pulse interval (days)	Total no. of band classes	No. of classes found in:		
		1 replicate	2 replicates	3 replicates
1	29	19	7	3
3	27	10	13	4
7	33	22	10	1
14	45	24	13	8

^a DGGE bands were scored in samples collected from 98 to 132 days.

TABLE 3. Phylogenetic association and physiological characteristics of isolates obtained from the different pulse intervals

Phylum and isolate ^a	Nearest GenBank relative	% Similarity	Accession no.	Growth kinetics when incubated in batch culture before transfer for:								Ectoaminopeptidase activity (nmol MUF h ⁻¹ ml ⁻¹ mg protein ⁻¹)
				1 day		3 days		7 days		14 days		
				Lag (h)	μ (h ⁻¹)	Lag (h)	μ (h ⁻¹)	Lag (h)	μ (h ⁻¹)	Lag (h)	μ (h ⁻¹)	
<i>Bacteroidetes</i>												
1 A-5 ^b	<i>Chryseobacterium</i> sp.	98	AY278484	0	0.57	0	0.54	1	0.42	2	0.40	1,273
1 A-9 ^b	<i>Chryseobacterium meningosepticum</i> ATCC 33958	99	AJ704543	0	0.57	1	0.53	3	0.34	3	0.35	2,236
1 C-3	<i>C. meningosepticum</i> ATCC 33958	99	AJ704543	0	0.42	0	0.40	3	0.23	2	0.23	2,232
3 A-6 ^b	<i>C. meningosepticum</i> ATCC 33958	99	AJ704543	0	0.48	0	0.42	3	0.36	2	0.41	2,952
3 C-3 ^b	<i>C. meningosepticum</i> ATCC 33958	99	AJ704543	0	0.41	0	0.35	3	0.29	3	0.38	2,711
7 C-2 ^b	<i>C. meningosepticum</i> ATCC 49470	100	AJ704544	0	0.57	1	0.58	1	0.41	0	0.48	2,590
3 A-13 ^b	<i>Flavobacterium aquatile</i>	94	M62797	0	0.53	0	0.41	3	0.32	3	0.34	764
3 B-3 ^b	<i>Flavobacterium</i> sp. strain 3A5	95	AF368756	0	0.36	0	0.35	0	0.21	0	0.19	488
7 A-6 ^b	<i>Flavobacterium</i> sp. strain 3A5	95	AF368756	0	0.33	1	0.32	1	0.42	2	0.18	536
7 B-4 ^b	<i>Flavobacterium frigoris</i>	94	AJ601393	0	0.38	0	0.36	1	0.23	1	0.21	330
1 B-1 ^{b,c}	<i>Sphingobacterium multivorum</i>	99	AB100738	0	0.49	0	0.48	2	0.40	3	0.35	1,095
1 B-8 ^b	<i>Sphingobacterium</i> sp. strain MG2	98	AY556417	0	0.51	1	0.5	2	0.33	5	0.33	1,127
1 C-2	<i>Sphingobacterium</i> sp. strain MG2	98	AY556417	0	0.42	1	0.43	2	0.33	2	0.42	1,071
3 A-3	<i>Sphingobacterium</i> sp. strain MG2	98	AY556417	0	0.33	1	0.38	2	0.33	2	0.28	1,074
7 A-7	<i>Pedobacter africanus</i> DSM 12126 ^T	98	AJ438171	0	0.45	2	0.42	3	0.38	4	0.34	210
7 A-12	<i>P. africanus</i> DSM 12126 ^T	99	AJ438171	1	0.63	4	0.56	5	0.33	4	0.30	299
14 A-12	<i>P. africanus</i> DSM 12126 ^T	98	AJ438171	0	0.13	1	0.33	3	0.24	3	0.23	416
14 C-13	<i>Flexibacter</i> sp. strain MDA 2495	95	AY238335	12	0.06	11	0.07	11	0.06	12	0.06	1,006
<i>Alphaproteobacteria</i>												
7 A-14	<i>Sphingomonas</i> sp. strain JSS-26	99	AF131296	3	0.58	2	0.32	3	0.27	4	0.20	167
<i>Betaproteobacteria</i>												
1 C-10 ^b	<i>Pseudomonas huttiensis</i> ATCC 14670 ^T	99	AB021366	0	0.37	0	0.39	3	0.29	2	0.34	2,181
<i>Gammaproteobacteria</i>												
1 B-3	<i>Stenotrophomonas acidaminiphila</i>	99	AF273079	0	0.38	1	0.40	2	0.32	3	0.37	119
3 B-1	<i>S. acidaminiphila</i>	99	AF273079	0	0.30	0	0.22	0	0.18	0	0.30	35
3 B-2 ^b	<i>Stenotrophomonas maltophilia</i>	99	AY445079	0	0.48	1	0.51	2	0.34	3	0.43	45
7 B-9	<i>S. acidaminiphila</i>	99	AF273079	0	0.57	0	0.65	3	0.58	2	0.62	2,482
7 B-2 ^b	<i>S. acidaminiphila</i>	99	AF273079	0	0.42	1	0.47	2	0.51	3	0.56	22
14 A-9 ^b	<i>S. acidaminiphila</i>	99	AF273079	0	0.30	1	0.29	3	0.22	3	0.21	19
3 C-10 ^b	Blackwater bioreactor bacterium BW3	99	AF394168	0	0.49	0	0.35	2	0.25	5	0.23	43
7 B-3 ^b	Blackwater bioreactor bacterium BW3	99	AF394168	0	0.38	0	0.38	3	0.30	2	0.28	32
14 B-1 ^b	Blackwater bioreactor bacterium BW3	99	AF394168	2	0.48	1	0.45	2	0.39	3	0.26	42
14 A-3 ^b	Blackwater bioreactor bacterium BW3	99	AF394168	0	0.35	1	0.33	1	0.43	3	0.24	39
<i>Actinobacteria</i>												
1B-14	<i>Arthrobacter</i> sp. strain R33S	97	AY572475	ND ^d	ND	2	0.22	2	0.23	4	0.19	42
7 A-1	<i>Arthrobacter mysorens</i> DSM 12798 ^T	98	AJ617482	0	0.45	1	0.46	3	0.27	4	0.23	126

^a Isolates are coded by the pulse interval from which they were isolated (1, 3, 7, or 14), followed by the replicate (A, B, or C) and isolate number

^b Mobility of PCR amplicon on DGGE matched that of a member of the mixed community.

^c Single isolates with two slightly different 16S rRNA gene sequences. The 1B1 second sequence matched 98% to *Sphingobacterium* sp. strain MG2 (accession no. AY556417), and the 3A3 second sequence matched 99% to *Sphingobacterium multivorum* (accession no. AB100738).

^d ND, not determined.

changes for the *Gammaproteobacteria* isolates which spanned the largest differences in pulse intervals did not reveal characteristics that might have been important selective forces.

Ectoaminopeptidase activity assays performed on each isolate indicated that those from the 1-day interval community had greater activity than those from the longer pulse intervals (Table 3). As previously found (26), the artificial substrate methylumbelliferone (MUF)-leucine does not react with the aminopeptidases found in *Gammaproteobacteria*. These analyses also revealed that the enzymes were localized on the cell surface. Surface association of proteolytic enzymes with the

producing bacterium should reduce the capacity for nonproteolytic cells to access hydrolysis products. The cell-associated activity ranged from 73 to 100% of the total activity.

DISCUSSION

Natural ecosystems are complex, dynamic environments in which the physical and chemical environments can vary in both time and space. Under these conditions, it is not surprising that many different microbes comprise the heterotrophic microbial community and that community composition is not stable. We

FIG. 5. Comparison of death rates (A) and lengths of stationary phase (B) after incubation in 10 mM Tris-HCl buffer for bacterial isolates. Each data point represents a discrete isolate obtained on day 90 from one of the systems.

created simple models of a planktonic aquatic ecosystem with less environmental variability, other than the provision of a single macromolecular substrate (protein) at defined time intervals. Protein is a typical representative of the polymeric substrates most abundant from decay of biomass in aquatic systems. With this controlled system, the effect of nutrient pulse intervals upon the dynamics of community composition, the physiological activity of the mixed community, and the physiological properties of bacterial strains selected under different periodicities could be determined.

The most intriguing result from these studies was the community dynamics observed over 130 days. At none of the pulse intervals was a stable community composition achieved. The results suggest that the dynamics and diversity of natural communities are not only due to the lack of constancy in the physical or chemical environment but also due to dynamic interactions between microbes. By evaluating the changes in community structure by using NMDS, we found that in all cases, the rate of community changes decreased after the first few weeks. This was not surprising because provision of a single polymeric substrate would rapidly select a subset of the diverse community that had developed on the broad range of organic substrates available in activated sludge used as our inoculum. However, even for the 1-day pulse interval communities, in which relatively small changes in community structure were observed after 25 days, the Dice similarity coefficients among fingerprint profiles from 25 to 132 days ranged from 0.97 to 1. Furthermore, the three replicates of each treatment were quite dissimilar to each other, exhibiting independent community dynamics throughout the experiment. After a pe-

riod of selection of 90 days, the Dice similarity coefficients of some replicates were <50% (Fig. 4).

What phenomena might explain the complex dynamics we observed, when natural selection would be expected to select the “fittest” strains? Theoretical analyses have explored the role of biological interactions in creating such dynamics. Competition for interactively essential resources generated oscillations in population density that led to “competitive chaos” (16, 17). Apart from resource competition, antagonistic interactions based on nontransitive rock-scissors-paper games have been postulated to promote biodiversity (5, 20). On the experimental side, investigations on the evolution of *Escherichia coli* during stationary phase (46) provide a genetic basis for the dynamics of bacterial communities. After less than 10 days of starvation, most *E. coli* cultures contained so-called GASP (growth advantage in stationary phase) mutants with a selective advantage over the wild-type strain, and continued starvation led to periodic sweeps of the population by new, fitter GASP mutants. If this phenomenon is found in the taxa present in our experiments, then the dynamics of mixed microbial communities under starvation may depend upon the stochasticity with which rounds of fitter mutants develop. The stochastic nature of these processes is consistent with our finding that replicate cultures exposed to the same pulse interval were dissimilar from each other. Although our work demonstrates the continued dynamics of mixed microbial communities, these experiments do not distinguish between physiological and genetic bases for these dynamics.

A second conclusion, derived from physiological analyses of replicate cultures subjected to identical nutrient pulses, is that microbial community functionality was conserved, even when community compositions were significantly different. The exponential growth rates and aminopeptidase specific activity levels of replicate mixed cultures exhibited less than twofold variation. Thus, although different phylotypes arose in different replicates, their performances in these systems were similar enough to result in communities whose functions were indistinguishable from each other. This implies a substantial degree of functional redundancy in these microbial communities, as has been found in a variety of natural and engineered microbial ecosystems (2, 10, 22, 38, 45). One rationale for the emphasis on analysis of microbial community composition in microbial ecology has been that determining composition is key to understanding community functionality. However, results from this and other recent studies suggest that the link is tenuous.

Nutrient regimens of different periodicities did result in mixed communities with distinct physiological properties. In particular, the nutrient-unrestricted exponential growth rates after nutrient addition ranged from 0.15 to 0.53 h⁻¹ and were inversely proportional to the pulse interval. It was surprising to find that in cultures pulsed every 7 days (in which the community was subjected to 6 days of starvation), no lag in growth was observed. Only in cultures pulsed every 14 days (with 12 days of starvation), was an initial period of submaximal growth observed. However, the kinetics differed from a true lag (non-growth) phase and were similar to the S-phase observed in mixed communities grown at very low rates in biomass recycle reactors (29).

The addition of a gelatin pulse did have modest effects on

the physiological state of the mixed cultures, with temporal dynamics in aminopeptidase, ATP, and RNA levels during the pulse. The aminopeptidase specific activities found in these pulsed cultures were of similar magnitude to levels found when gelatin was continuously added to a chemostat (2a). *Cytophaga johnsonae* had a decrease of 50% in ATP content when shifted from nutrient excess to starvation (14), and starved *Rhodobacter palustris* had a decreased RNA content (35). Some bacteria can maintain high levels of RNA content under starvation; Koizumi et al. (21) suggested that this was essential for immediate macromolecular synthesis when substrates are supplied. Even after starvation, RNA levels in our mixed communities were relatively high (25 to 80 μg RNA mg cell protein⁻¹); this could be a factor in the observation of immediate exponential growth by the communities in response to a nutrient pulse.

The physiological characteristics of bacteria isolated from each system were examined to test the hypotheses of Konopka (24) that different pulse intervals would alter the selection for bacteria with low death rates under starvation or with a rapid resumption of growth after nutrient addition. The hypotheses were not supported by the physiological analyses. Bacteria from cultures given nutrients every 14 days did not have significantly lower death kinetics than those provided with nutrients each day. Lags in growth of bacteria isolated from different nutrient pulse regimens were not distinguishable; in general lags were short even when the period of nutrient deprivation was long. This suggests that if the bacteria underwent a starvation response that produced dormancy, it could be very quickly reversed. The inadequacy of the model of Konopka (24) reflects its simplicity: it compared the relative fitness of an organism that underwent a starvation response to that of an organism that did not, and none of the complex community dynamics observed in our experimental systems were reproduced.

The most intriguing result obtained from analysis of the growth dynamics of isolates was that previous history (length of starvation phase) influenced growth kinetics after nutrient addition. The unrestricted growth rates measured after 1 day of starvation paralleled the results found in the community dynamics; bacteria selected in systems pulsed every day had higher growth rates than those obtained from systems pulsed every 14 days. However, if strains from all treatments were starved for the extended period of 14 days, the growth rates of many converged toward those observed in communities pulsed every 14 days. The physiological basis for these observations is unknown; some theoretical work has been done to assess the effects of adaptation on kinetic constants (44), but not under the extreme changes in starvation time imposed here. These results leave unclear the extent to which the differences in mixed-community functionality (growth rates) between frequent and infrequent pulse intervals were due to selection for genotypes with particular growth rates (for example, due to differences in rRNA operon copy number [39]) as opposed to the physiological consequences of pulse interval upon subsequent growth. In the specific case of strain 1A9, the effects appeared to be physiological, as the slow growth response to nutrients found after 14 days of starvation was replaced by a higher growth rate when nutrients were provided again 1 day later. These physiological changes add additional complexity to understanding the role that stochastic events (such as the oc-

currence of fitter mutants) play in the dynamics of community structure. This would be particularly true in natural systems, where the length of the interpulse interval may not be constant.

Although the physiological analyses of the isolates did not provide a basis for understanding the selective forces that operated at different pulse intervals, they do suggest that the physiology of starvation recovery should be analyzed as completely as the response to starvation has been. Starvation conditions produce changes in gene expression that promote starvation survival (34). Survival time is often related to the level of reduction in cell metabolism (11). There has been much less experimental work on the recovery from starvation. After 10 days of starvation, *Rhizobium leguminosarum* bv. Phaseoli immediately initiated macromolecular synthesis after nutrient addition, but cell division was not observed for 10 h (42). A number of the isolates (as well as the mixed communities) exhibited a remarkable capacity to rapidly resume growth after extended periods of starvation.

Phylogenetic analysis of the isolates suggested that some taxa have the versatility to succeed under a broad range of nutrient supply rates. This was particularly true for the *Gamma-proteobacteria*, in which clusters of similar isolates arose from either three or four of the pulse intervals. Clusters of related strains from the *Bacteroidetes* were restricted to either the 1- and 3-day or the 7- and 14-day pulse intervals. The differences in physiological traits among these isolates could not be related to differences in selective forces expected between short and long nutrient pulse intervals. In natural systems, differences in physiological characteristics among strains with identical 16S rRNA sequences have been documented (18), although the ecological significance of those differences was not shown. Relating the specifics of microbial community composition to functional performance is a difficult challenge; at scales of ecology (the community level) where the numbers of interactions among organisms and their environment are large, predictive power is usually low (30). The dynamic changes and substantial diversity in microbial community composition that we found when the environment was predictable (if not constant) indicate that the interplay between microbes may be equally as important as the selective forces of the external environment in structuring microbial communities.

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