Strain and Function Stability in Gnotobiotic Reactors

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Abstract. The ability of the community level physiological profiling approach to analyzing microbial community structure was examined in a constructed community. Four bacterial strains, characterized by BIOLOG®-GN profiles, were combined into 2 communities which were maintained for 7 weeks on 2% wheat residue in distilled water with 10% daily removal and replenishment of medium. after 3 weeks incubation, aeration was stopped for 5 days. Although the abundance of all the isolates decreased during the perturbation, all the strains persisted throughout the experiments. Only 80% of functions lost during perturbation were recovered afterward. Persistence of strains in a community did not guarantee persistence of metabolic functions which those strains could perform.

The approach to community structure showed that simple summation of pure culture functional profiles did not accurately predict the functional profiles of the mixed communities. Functions resulting from synergistic interaction between the strains appeared in the mixed communities, but were inactive during the perturbation. The assay plates could not detect metabolic activities of strains present at densities below $\approx 1 \times 10^7$ CFU ml^{-1}. Population densities of component strains in the community could not be inferred from community-level functional profiles. Because strains behaved differently in mixed and pure cultures, community behavior was not accurately predicted by pure culture performance of even the densest strains in the communities.

Keywords. Bacteria, community structure, stability, resiliency

1. Introduction

The recognition that the behavior of communities of microorganisms in the environment is not well predicted by studies of pure cultures in laboratory situations has led to a flurry of effort to detail the structure and function of the communities and their constituents. Studies fall into two broad categories: one includes those investigations concerning the genetic or taxonomic structure of the community, and the second focuses on the functional characteristics of the community and its members. The use of molecular techniques offers insight into

the genetic composition of the organisms that constitute the community, and the recent efforts to extract RNA and DNA from whole communities extend that insight to the intact community. Some of the molecular techniques also have promise for examining functional aspects of the microbial communities, especially if probes for loci that code for enzymes of importance are available. The use of the physiological profiles, specifically, those dealing with sole carbon source utilization (Garland, 1996; Garland and Mills, 1991; Insam *et al.*, 1996; Winding, 1994; Zak *et al.*, 1994), is one of several techniques that should be considered as a means of judging similarity among microbial communities (Turco *et al.*, 1994)

The use of physiological profiles offers another view of the functional abilities of microbial communities. Most often, the profiles are based on use of a series of organic substrates as sole sources of carbon, energy, and electrons. The use of commercially prepared test kits such as the BIOLOG® or PHENE-PLATE® assays can make the extensive testing necessary much easier as well as making the results more comparable from study to study. Questions arise, however, in using the commercial preparations with regard to reproducibility and interpretation.

We have conducted a study of a constructed communities in an effort to examine specifically, some of those questions. The use of a small number of isolates in a constructed community permitted the meaningful evaluation of the physiological profile approach to community structure in the context of determining if the assay can be used to examine population changes in a dynamic community, and if the approach produces reproducible results when applied to similar or identical samples. Furthermore, we sought to determine if the response patterns in the whole community reflected a summation of the properties of the individuals. Answers to each of these questions are essential to the use of the approach in microbial community ecology.

2. Methods and Materials

2.1 General approach

Detailed methods for isolation of the test strains, construction of the reactors, and conduct of the experiments are given in Bouma (1995) and Bouma and Mills (submitted). A brief description of the supporting work is presented here.

Four bacterial strains used as building blocks for the gnotobiotic community capable of degrading inedible plant material were isolated and functionally characterized. The strains were assigned numbers (6a, 9, 13a, and 19), and BIOLOG®-GN profiles were obtained, but no additional attempt was made to assign a conventional name to the organisms. For enumeration of separate population densities in mixed culture, strains with distinct colony morphologies were chosen. The strains were functionally diverse in their ability to degrade the different components of inedible (human) plant matter and had distinguishable functional profiles on BIOLOG®-GN assay plates (Bouma, 1995). Pure culture

growth patterns, both population densities and functional activity profiles, were determined for comparison with observations from mixed culture.

The four chosen strains were combined into parallel gnotobiotic communities in sterile, aerated 3-liter reactor vessels. Mixed cultures were maintained for 6 to 7 weeks, with daily removal of one tenth of the culture volume and replenishment with an equal volume of fresh sterile medium containing inedible plant material. Every effort was made to prevent recruitment of new strains (contaminants) into the communities.

Population dynamics of the communities were determined by spread plate enumeration of viable cells on TSA plates at least three times a week. Functional assays if each community's ability to degrade the 95 different compounds on the BIOLOG®-GN plates were performed once a week. Data obtained from simultaneous monitoring of both population and functional dynamics through a perturbation (anoxia) allowed examination of the relationship between population structure within a community and functional behavior of that community.

2.2 Production of physiological profiles with BIOLOG®-GN plates

Since the goal of the physiological profiles of the mixed community was to monitor the functional activity of the mixed liquid culture, the standard BIOLOG® protocol had to be modified. An additional complication was that part of the organic substrate in the liquid medium in this study was present as particulate matter. For the BIOLOG® assay to be successful, all extraneous carbon sources must be removed from the bacterial inoculum added to the assay plate. The ability of an isolate to be cleanly separated from the particulate matter in 2% wheat liquid medium, resuspended in saline, and then to produce a BIOLOG® response in a timely fashion was the first hurdle for candidate members of the mixed community.

A uniform protocol was established for extracting cells for both pure and mixed culture functional profile determinations. A 100 to 150-ml sample of liquid culture was placed in a sterile 500-ml Erlenmeyer flask, shaken vigorously for 15-20 seconds, and allowed to settle for 2-4 min. Carefully avoiding the solid residue at the bottom of the flask, approximately 90 ml of liquid was removed to a sterile 250-ml flask, and allowed to settle for another 15 min. Equally-sized 35 to 37-mL aliquots of the cloudy golden brown liquid were pipetted into two sterile 50-ml polypropylene centrifuge tubes, again avoiding any solid residue settled on the bottom of the source flask. The tubes were capped and spun for 18-20 minutes at 27,100 × *g*. The clear brown supernatant was decanted and discarded. The cells forming the top layer of each pellet were resuspended in 10 to 15 ml of sterile 0.85% NaCl. The total volume of saline (20 to 30 ml) used to resuspend both pellets of a sample varied inversely with the suspected density of cells in the sample (density was estimated from the plate-count enumeration history of that strain or culture and the size of the cell layer in the pellet). The standard volume of resuspended cells was 30 ml.

Duplicate 100 µl samples of the resuspension were diluted through parallel series and spread onto TSA plates for enumeration of CFU ml^{-1}. Five replicate 1:

10 dilutions of the original resuspension were created by pipetting 2.5 ml of the original resuspension into 22.5 ml of sterile 0.85% saline. Each replicate was used to inoculate a BIOLOG®-GN plate with 150 µl of diluted resuspension per well. The assay plates were incubated in the dark for 24 h at 24-27°C. The 24-h incubation period was chosen because there was usually no visible pattern at 4 h and 24 h is regarded as the upper limit of the "active" functional profile, as opposed to the "potential" physiological profile obtained with longer incubations (Garland and Mills, 1991). At the end of incubation, assay plates were placed in plastic bags and refrigerated for up to 3 weeks before being read in a microtiter plate reader. Because the functional profiles determined by this cell extraction procedure were not obtained by following the standard procedure for reliable strain identification (Marello and Bochner, 1989) these profiles should not be compared to those in the BIOLOG® database without risk of misidentification.

Two to eight replicate profiles were obtained and compared to establish the profile at 24 h for each strain in pure culture and also for each sample from the mixed culture experiments. All analyses were done with binary response data (+ or -) obtained by subtracting 140% of the control well's absorbance (Marello and Bochner, 1989) from each well. With that adjustment, positive absorbance values were considered indicative of substrate metabolism, and negative values were considered to indicate no metabolism. Binary patterns were of primary concern in this study because the presence or absence of detectable levels of metabolic activity is the first step in relating population dynamics to the dynamics of metabolic functions. The degree of color development in sample wells is most useful in longer duration studies (Garland and Mills, 1991; Haack *et al.*, 1995). Interval-level absorbance data (i.e., the degree of color development in each of the wells) were used in this study to determine the mean absorbance of substrates giving equivocal responses (for substrate-sample combinations that did not give uniform, e.g. 5/5, results, or for weak color development).

2.3 Application of the perturbation to the community in the bioreactor

In the first experiment, the initial aeration rate ranged between 500 and 800 ml min^{-1}. The community was stressed by ceasing aeration for one week, starting one hour after the Day 22 samples for density and functional profile were removed. At Day 24 an additional functional assay was performed. Minutes after the Day 29 samples were taken, aeration was restarted at the initial rate. Starting at Day 40, aeration became increasingly intermittent and gradually declined in flow rate due to blockage of the outflow line. The outflow air line had to be repeatedly tweaked to reopen an air passage. During the final week of the experiment, the flow rate during bouts of aeration averaged only 400 to 450 ml min^{-1}.

In the second experiment, the initial aeration rate was 400 ± 200 ml min^{-1}. Minutes after the Day 24 removal and replenishment of medium, the community was stressed by ceasing aeration for 5 days. No functional profile sample was removed at Day 24, but an additional functional assay was performed at Day 26, two days into the perturbation. Minutes after the Day 29 density and functional activity samples were taken, aeration was resumed with an air line connected to a

new laboratory compressed air supply. A stable aeration rate of about 400 ml min^{-1} was established and maintained until Day 34, when the aeration rate was adjusted to 950-1150 ml min^{-1} after each day's medium removal and replenishment, and ranged between 650-1200 ml min^{-1} over the course of a day. Aeration remained good in the second run until the final day, when the aeration rate fell to 55 ml min^{-1}, at which time the experiment was terminated.

3. Results

3.1 Test reproducibility

To examine the reproducibility of the BIOLOG® assay with the strains of bacteria used in the study, several replicate plates for each strain were inoculated, incubated for 24 h, and read. The binary profiles produced from the replicates were highly reproducible (Table 1). Strain 19 produced the most tests not in complete agreement, with 6 of the 95 compounds producing mixed results. Of the six deviations, 3 had 4/5 plates positve, and 3 had 3/5 plates positive. The other three strains produced a total of 4 non-unanimous results; two tests (1 from strain 9 and 1 from strain 13) had 3/5 plates positive, a single test from strain 13a had 2/5 positive and 1 test from strain 6 had a single plate positive (1/2). In summary, out of 380 total tests replicated as indicated in Table 1, only 10 (2.6%) produced equivocal results. The non-unanimous test results were not clustered within any group of compounds, but were spread throughout the 95 substrates.

Table 1. Tests from BIOLOG®-GN plates in which disagreement among replicates was observed. The remaining 85 of the 95 tests were all in agreement at the level of replication noted

Compound	Strain (No. of replicates)			
	6A (2)	9 (5)	13A (5)	19 (5)
α-cyclodextrin				3
dextrin				4
maltose				4
xylitol				4
α-hydroxybutyric acid				3
p-hydroxyphenylacetic acid		3		
L-proline			3	
L-serine			2	
L-threonine				3
thymidine	1			

In the mixed culture incubations, 9 sampling times produced 855 total tests, each of which was replicated 5 times. In the first incubation, 39/855 tests (4.6%) were not in complete agreement (Table 2). The second incubation had only 16/855 tests (1.9%) that did not agree.

Table 2. Test reproducibility in the mixed communities from the bioreactor incubations. Note that only the tests which were not in complete agreement are shown. All other tests were positive in all replicates at each sampling time or negative in all replicates at each sampling time

Compound	Days in Mixed Culture								
	1	8	15	22	24	29	36	43	50
Tween 80							4		
N-acetyl-D-galactosamine					1				
L-arabinose			4						
L-fucose				1	3			3	
lactulose						4			
D-mannitol				4					
L-rhamnose			4				2		2
mono-methyl succinate		4							
acetic acid		4							
D-gluconic acid			3					3	
D-glucuronic acid	1								
α-hydroxybutyric acid			2						
p-hydroxyphenylacetic acid									3
D,L-lactic acid									2
propionic acid			1		1			2	
succinic acid					1				
L-alanyl-glycine		1	4						
L-proline			2		3				1
L-pyroglutamic acid					1				
L-serine		3							1
L-threonine		2			3			2	
putrescine					2				1
bromosuccinic acid				3					
urocanic acid			1					1	

3.2 Population dynamics and community-level physiological profiles

During the bioreactor incubations, the numbers of cells of the various strains reached an approximate steady state level that was slightly different for each strain (Fig. 1). Strain 19 did not reach the levels of abundance of the other strains except at the very beginning of the experiment. Strain 6A, on the other hand,

grew to levels that approximated those of strains 13A and 9, but did so only after about a week of incubation. During the perturbation, the numbers of cells of all of the strains seemed to drop slightly, but those of strain 19 fell by more than an order of magnitude. The die-off of strain 19 in this reactor lagged behind the cessation of aeration and the organism recovered as soon as aeration was reapplied to the reactor.

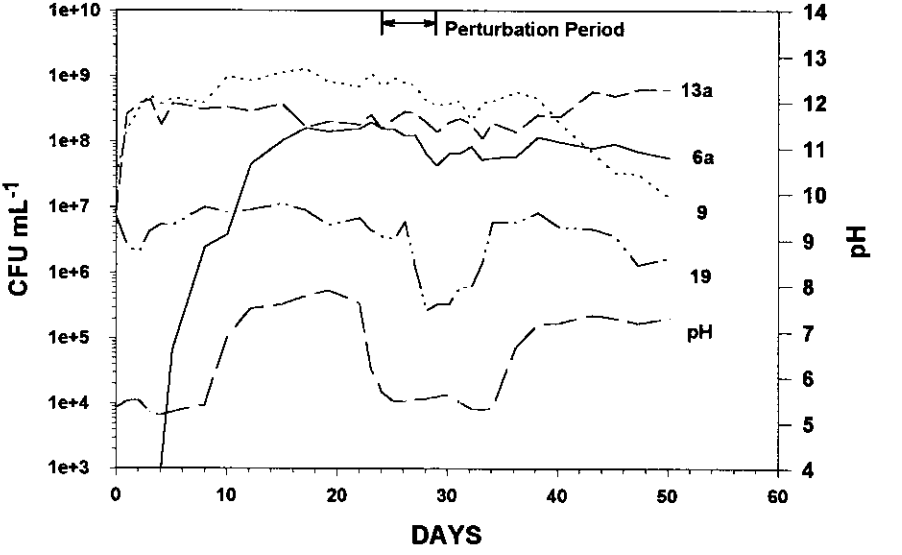

Fig. 1. Dynamics of the inoculated strains in the first bioreactor run. Note that the decrease in countable numbers of strain 19 lagged behind the onset of the perturbation of anoxia

The substrate utilization patterns showed that several of the compounds stopped being metabolized during the perturbation, and that activity did not reappear in all cases after the air was turned on again. Not all were recovered after the reapplication of air to the reactor. For example, metabolism of acetic acid, D-gluconic acid, α-hydroxybutyric acid, p-hydroxyphenylacetic acid, several other carboxylic acids, glycerol, putrescine, and several amino acids ceased during the perturbation, but recommenced after the anoxic period. In still other cases, mostly in the carbohydrates, polymers and esters, activity was present throughout the incubation, including the period of perturbation.

Metabolism of N-acetyl-D-galactosamine, succinic acid, L-alanyl-glycine, L-pyroglutamic acid, and bromosuccinic acid was observed before the perturbation in the bioreactor incubation, but no activity was present after reapplication of the air supply. Because the original isolates were detectable at all times in the reactor, we infer that the genetic makeup of the community did not change so drastically over the course of the experiment to account for the change in the spectrum of compounds metabolized.

3.3. Comparison of community-level physiological profiles with isolate profiles

The number of sole-carbon substrates metabolized by microbial communities changed over time. In the first incubation, the number of degraded carbon sources increased monotonically until the time of the perturbation, and showed a second increase between the end of the perturbation on day 29 and the end of the reactor run. (Fig.2) Due to the slight differences in the binary patterns on the replicate assay plates, the average of the sum of positive responses for each plate peaked at only 44 on day 22, although the total number of active functions was 47 on day 22. According to both measures, the number of active functions dropped to 21 by the end of the perturbation. After the return of aeration, the number of detectable metabolic functions gradually increased to 38. D-glucuronic acid was not degraded in pure culture by any of the isolates, was metabolized at a barely detectable level on the first day, and utilization was never detected in this community again. Five other compounds were also degraded by synergistic interaction among the strains: N-acetyl-D-galactosamine, L-fucose, L-rhamnose, D-gluconic acid, and L-alanylglycine.

In the second run of the mixed community (data not shown), the same 6

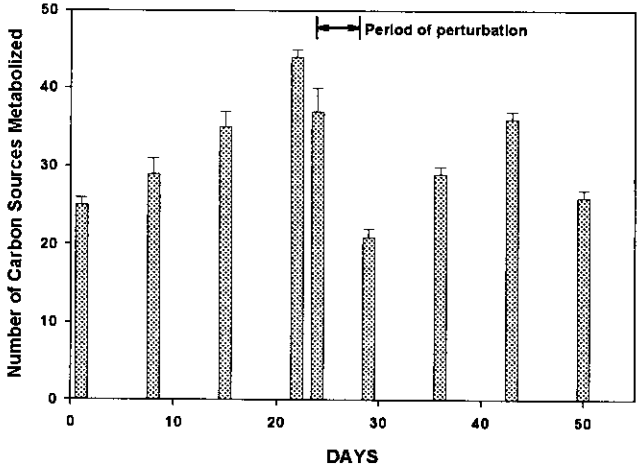

Fig. 2. Changes in the number of positive tests during the course of the first community run in the bioreactor

compounds not degraded by any of the isolates in pure culture were metabolized synergistically by the community, and two additional compounds hydroxy-L-proline and glucose-1-phosphate also were metabolized by the community, although glucose-1-phosphate use was seen only on the first day of the incubation

and only in 2 of 5 plates. Of the 30 metabolic activities detected on the first day of the second reactor run, 8 disappeared permanently after the first week. Seven of the 8 affected compounds were synergistically degraded: N-acetyl-D-galactosamine, L-fucose, L-rhamnose, D-glucuronic acid, L-alanyl-glycine, hydroxy-L-proline, and glucose-l-phosphate. Observing the behavior of the community in the first reactor run with respect to those 8 compounds did not clarify possible trends: 2 of the 8 functions never appeared (synergistic metabolism of glucose- 1 phosphate and hydroxy-L-proline), three functions were permanently lost after the perturbation (degradation of N-acetyl-D-galactosamine, L-alanyl-glycine, and D-glucuronic acid), and three were lost and eventually recovered (L-fucose, L-rhamnose, and L-serine).

4. Discussion

The physiological profiling approach to community structure offers a reliable means of examining communities with regard to the arrangement of functional capability of the assemblage. Tests with the BIOLOG® plates were perfectly consistent within replicates in 95% - 99% of all the tests run (2080 for the entire study). While some variability is encountered in dealing with environmental samples (Garland and Mills, 1991; Garland and Mills, 1994), that variability may realistically describe the small-scale patchiness in the distribution of microbes and their functions. Indeed, the variability in environmental samples may prove to be helpful in determining the realistic scales of variability in distribution of microbes that constitute communities.

Use of short-term (i.e., less than 24 hour) incubations of the test plates provides a profile that is more reflective of *in situ* activity, or at least of constitutive activity, possessed by a high proportion of the organisms present in the test well, at least when binary data are considered, because bacteria that utilize wide ranges of carbon and nitrogen sources generally synthesize the appropriate enzymes when they are needed (Mahan *et al.*, 1993). Concerns over the lack of reproducibility of profiles can be overcome by use of binary coding of the data (which minimizes quantitative differences in profiles) combined with replication of the profiles. In natural communities which contain much larger numbers of different organisms than employed in these experiments, one might expect a greater variability of response, but the use of binary data coupled with the threshold cell concentration ($\approx 10^7$ cells ml^{-1}) to effect a visible response in the test well, suggests that variability will be minimal if the sampling scale does not exceed the correlation length scale of the community, i.e., replicate samples are collected from within a single community.

As pointed out by Bouma and Mills (submitted), the signature of a community subjected to a physiological profiling with sole carbon source utilization is not the sum of the characteristic patterns of the individual community members.

Physiological profiling with sole carbon source utilization can be used to demonstrate time-dependent properties of communities. In the present study, several of the substrates metabolized by the communities in the early part of the

incubation disappeared during the anoxic period. In some cases those substrates were again metabolized after the resumption of aeration, but others were not. The observation that some of the metabolic capacity of the community was lost permanently is of importance, because none of the organisms inoculated into the reactors became extinct. This behavior is somewhat surprising, as the substrate-nutrient mixture was the same throughout the entire incubation. We cannot determine what caused the permanent loss of the characters.

The ability to detect synergistic relationships among the community members is an important property of the physiological profiling approach to community structure that is not shared by any of the other techniques that are currently applied, including the most sophisticated molecular methods. Neither genetic analysis nor fatty acid profiles can categorize the community on the basis of interactions, either those currently occurring *in situ*, or those potentially present as a result of the activities of the organisms. Perhaps the most powerful community descriptions are those that employ genetic profiling using selected molecular techniques in combination with physiological profiling using approaches such as described here.

5. References

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