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Analysis of Factors Affecting the Accuracy, Reproducibility, and Interpretation of Microbial Community Carbon Source Utilization Patterns†

SHERIDAN KIDD HAACK,[1]* HELEN GARCHOW,[1,2] MICHAEL J. KLUG,[1,2,3] AND LARRY J. FORNEY[1,3]

Center for Microbial Ecology,[1] Department of Microbiology,[3] and Kellogg Biological Station,[2] Michigan State University, East Lansing, Michigan 48824

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We determined factors that affect responses of bacterial isolates and model bacterial communities to the 95 carbon substrates in Biolog microtiter plates. For isolates and communities of three to six bacterial strains, substrate oxidation rates were typically nonlinear and were delayed by dilution of the inoculum. When inoculum density was controlled, patterns of positive and negative responses exhibited by microbial communities to each of the carbon sources were reproducible. Rates and extents of substrate oxidation by the communities were also reproducible but were not simply the sum of those exhibited by community members when tested separately. Replicates of the same model community clustered when analyzed by principal-components analysis (PCA), and model communities with different compositions were clearly separated on the first PCA axis, which accounted for >60% of the dataset variation. PCA discrimination among different model communities depended on the extent to which specific substrates were oxidized. However, the substrates interpreted by PCA to be most significant in distinguishing the communities changed with reading time, reflecting the nonlinearity of substrate oxidation rates. Although whole-community substrate utilization profiles were reproducible signatures for a given community, the extent of oxidation of specific substrates and the numbers or activities of microorganisms using those substrates in a given community were not correlated. Replicate soil samples varied significantly in the rate and extent of oxidation of seven tested substrates, suggesting microscale heterogeneity in composition of the soil microbial community.

Garland and Mills (2) recently introduced the use of community-level carbon source utilization patterns for comparison of microbial communities from different habitats. They used commercially available microtiter plates that contain 95 carbon substrates (Biolog GN; Biolog, Inc., Hayward, Calif.), which they directly inoculated with environmental samples from freshwater, saltwater, estuarine, and hydroponic solutions, from the rhizosphere of hydroponically grown wheat, and from soils. Similarly, Winding (5) analyzed the whole-community Biolog GN substrate utilization profiles of samples taken from several types of forest soils, as well as from size class fractions within one soil type, and Zak et al. (6) studied the profiles of soil samples from six desert plant communities along an elevational and moisture gradient. In all three studies, multivariate statistical methods were used to establish distinctions among the resulting community substrate utilization profiles and thereby classify the community samples with respect to their carbon source metabolism. Garland and Mills (2) proposed that since separation of community Biolog profiles by multivariate analyses is based on differences in carbon source utilization between samples, it provided "a functional basis to distinctions among communities." All three groups suggested that in addition to establishing ecologically relevant (e.g., soil versus freshwater) classifications of microbial communities, substrate utilization profiles might offer information with regard to community function (2), metabolic potential (5), or functional diversity (6).

To interpret the functional diversity or metabolic potential of microbial communities based on Biolog patterns of substrate utilization, it is important to understand factors which affect substrate utilization when Biolog microplates are inoculated with mixtures of microorganisms. In principle, the Biolog assay is done by colorimetrically measuring tetrazolium dye reduction that is coupled to substrate oxidation. The degree to which each of the 95 substrates is oxidized is determined after a fixed incubation period. A positive response is identified as an absorbance or optical density value greater than that occurring in the blank well. If the values for all substrate-containing wells are analyzed by multivariate statistics, two types of distinctions between samples can be made. First, the presence or absence of a positive response to each of the 95 substrates or to groups of substrates (e.g., polymers) can be recorded. Second, when the same substrates are utilized by different communities, samples with consistently high values for certain substrates can be segregated by multivariate statistical procedures from those with consistently lower values for the same substrates. For example, Zak et al. (6) distinguished desert soil microbial communities by both methods.

It is tempting to assume that differences in patterns of Biolog substrate oxidation by different communities represent real differences in the numbers or types of microorganisms in these communities and that these differences reflect the activities expressed in the environments from which the samples were obtained. However, Garland and Mills (2) and Winding (5) both found that variation in response from one sample to another was not necessarily a function of structural differences in the communities. Instead, both noted a strong correlation between inoculum cell density and the rate of color development, so that community differences in substrate oxidation

* Corresponding author. Present address: Water Resources Division, U.S. Geological Survey, 6520 Mercantile Way, Suite 5, Lansing, MI 48911-5991.

† Contribution 786 of the Kellogg Biological Station.

recorded at a fixed time might simply reflect differences in the total number of microorganisms in the communities. Moreover, both groups found that color development appeared to depend on cell growth in substrate-containing wells, so that it was impossible to determine if all members of the community capable of utilizing the compound had contributed to the profile or if the response in a given well resulted from growth and activity of a subset, or single member, of the community. Zak et al. (6) acknowledged these limitations but argued that substrate utilization profiles nonetheless provide a "rich data set" for studies of the function and diversity of microbial communities. However, Garland and Mills (2) suggested that further research was required to determine whether community-level carbon substrate utilization patterns were repeatable and to determine the causes of differential substrate oxidation among samples.

The research described here was conducted to determine factors which influence substrate oxidation when Biolog microplates are inoculated with samples from microbial communities. We used soil bacterial isolates, model bacterial communities composed of combinations of these strains, and actual soil microbial communities. We wished to determine, for axenic cultures and model communities, (i) whether there was a relationship between growth in wells during microplate incubation and substrate oxidation, (ii) the influence of inoculum density on the rate of substrate oxidation, (iii) whether the rate and extent of oxidation of a given substrate were reproducible when inoculum density was controlled, and (iv) whether the pattern of positive and negative responses to all 95 substrates in Biolog microplates was reproducible. Using two model soil bacterial communities for which the taxonomic structure and carbon substrate utilization patterns of the individual members were known a priori, we addressed whether Biolog profiles of whole communities reflected the taxonomic structure of those communities and whether we could detect contributions of individual populations to the whole-community Biolog profile. Finally, we examined community-level profiles of actual soil microbial communities and interpreted the results of multivariate statistical analyses of model and actual soil microbial community responses in light of knowledge obtained from the work with isolates. Our results verify the utility of this approach for the classification of microbial communities and provide information on factors which influence substrate oxidation and must be taken into account when interpreting the functional significance of whole-community substrate utilization profiles.

MATERIALS AND METHODS

Bacterial isolates. Ten bacterial isolates were obtained from soils of the Long Term Ecological Research site at the Kellogg Biological Station, Michigan State University. These isolates were identified, by phenotypic characteristics and fatty acid profiles (3) as two *Bacillus* spp., two *Arthrobacter* spp., two *Pseudomonas* spp., three *Cytophaga* spp., and one *Corynebacterium* sp. All cultures had been isolated on R2A agar (Difco, Detroit, Mich.) and maintained on R2A agar slants in the refrigerator with regular subculturing. These 10 strains were routinely cultured at 28°C on R2A agar or in the corresponding broth medium that lacked agar (designated R2A broth). Broth cultures were aerated by shaking. Standard curves correlating cell number (obtained by acridine orange direct counts) and biomass (micrograms [dry weight] per milliliter) with optical density at 590 nm (OD_{590}) were obtained for each strain during growth in R2A broth at 28°C and were used for estimation of cell number or biomass in all experiments where indicated. Viable cell counts were determined by plating on R2A agar.

Biolog substrates. For all studies reported herein we used the Biolog, Inc., gram-negative (GN), gram-positive (GP), or no substrate (MT) 96-well microplates. Each of the 95 substrate wells in standard Biolog GN and GP microplates contains (in dried form) a complex, low-concentration, buffered nutrient medium (1), a tetrazolium redox dye, and a carbon substrate. The substrate blank well contains the nutrient medium and dye but no substrate. The GN substrates were divided by Garland and Mills (2) into 11 categories: polymers (substrates 1 to 5), carbohydrates (substrates 6 to 33), esters (substrates 34 and 35), carboxylic acids (substrates 36 to 59), brominated chemicals (substrate 60), amides (substrates 61 to 63), amino acids (substrates 64 to 83), aromatic chemicals (substrates 84 to 87), amines (substrates 88 to 90), alcohols (substrates 91 and 92), and phosphorylated chemicals (substrates 93 to 95). GP microplates contain 62 of the same substrates as GN microplates plus 33 different substrates in similar categories.

MT microplates contain the dried nutrient medium and redox dye but no substrate in any well. Stock solutions (0.5%, wt/vol) of glucose, galactose, L-asparagine, aspartate, cellobiose, mannitol, and L-serine (all from Sigma Chemical Co., St. Louis, Mo.) were prepared in 50 mM phosphate buffer (pH 7) and sterilized by passage through a sterile 0.22-μm-pore-size filter (Millipore Corp., Bedford, Mass.). Stock solutions were dispensed to MT microplate wells in 75-μl aliquots. Blank wells received 75 μl of buffer.

Microplate protocols. Pure-culture inocula for microplates were typically prepared in one of two ways. Cultures grown on solid medium for 24 h were swabbed from the agar surface and resuspended in 50 mM phosphate buffer (pH 7). Cultures grown in broth medium were centrifuged (10,000 × *g*, for 10 to 15 min), the supernatant was removed, and the cells were resuspended in 50 mM phosphate buffer (pH 7) and held for 1 h before inoculation of microplates. Biolog GN or GP microplate wells were inoculated with 150 μl of these cell suspensions that had been adjusted to the appropriate density (GN, $\sim 3 \times 10^8$ cells per ml, GP, $\sim 4.5 \times 10^8$ cells per ml) by comparison with the turbidity standards supplied by the manufacturer. MT plates were inoculated with cell suspensions having an OD_{590} of ~0.45. Isolates, combinations of two or three isolates, and buffer were added to MT microplate wells such that the total volume of inoculum equalled 75 μl but the number of cells of each isolate in each well was equivalent. All MT plate wells also contained 75 μl of substrate solution or buffer (blank).

Inocula for model communities were obtained by growing cultures of the individual bacterial strains in R2A broth at 28°C for approximately 16 h. The OD_{590} after 16 h was correlated with cell count (cells per milliliter) or biomass (micrograms [dry weight] per milliliter) from standard curves previously established for each bacterial strain. Culture volumes required to achieve specific proportions of bacterial strains were combined and centrifuged to obtain the "community" cell pellet, which was resuspended in 50 mM phosphate buffer (pH 7) for 1 h prior to inoculation of GN microplates. Communities were designed such that the final cell density (total of all bacterial strains in the community) would fall within the recommended range for inoculation of Biolog GN microplates.

Three bacterial strains were used to determine if substrate oxidation was related to cell growth following inoculation. Eight replicate wells of Biolog MT plates that contained 0.25% (wt/vol) galactose as substrate were inoculated with each bacterial strain. The initial cell density was either $\sim 10^8$ or 10^7 cells per ml. Color development (OD_{590}) was recorded at 1-h intervals for 24 h and then once again at 48 h. At 1, 3, 6, 9, 19, 25, and 48 h, one of the eight replicate wells for each bacterial strain or combination was sampled to determine viable cell count.

Microplates were incubated at 28°C and were read at 590 nm in either the Biolog microplate reader with Microlog 2N software or the EL312e Kinetics reader (Bio-Tek Instruments, Inc., Winooski, Vt.) with Kineticalc II software. In the standard Microlog 2N software supplied by the manufacturer, a positive result must be of higher OD_{590} than the substrate blank reading and must be at least 40% of the highest positive substrate response for that microplate. This threshold was used throughout our study for assignment of a positive or negative response for all GN or GP substrates. The Bio-Tek EL312e reader and Kineticalc II software were used to obtain kinetic readings of color development in MT plates. Blank wells were assigned to sectors of each microplate as appropriate, and the reading at 590 nm of each blank well was subtracted from the reading of each substrate-containing well in that sector, at each reading time.

Environmental effects on isolate Biolog GN profiles. At least seven independent Biolog GN profiles were obtained at 24 and 72 h for each bacterial strain following growth in R2A broth at 28°C. The response to each substrate (i.e., always positive, always negative, or variable over the seven replicates) was determined. These reference profiles were used as the standard against which effects of growth medium, growth temperature, and inoculum density, as well as the results of our model community experiments, would be assessed. Biolog GN profiles were then obtained for each of the 10 bacterial strains grown under the following conditions: (i) on nutrient agar, tryptic soy broth agar (tryptic soy broth solidified with 3% [wt/vol] agar) (both from Sigma Chemical Co.), or R2A agar at 28°C and (ii) in R2A broth at 17 and 32°C. In the latter case, Biolog microplates were incubated at the growth temperature. Each profile was determined in triplicate and readings were obtained at 24 and 72 h. Profiles were also obtained (three replicates; 0-, 4-, 7-, 11-, 24-, and 96-h readings) for each bacterial strain after inoculation to GN microplates at 0.1 times the recommended cell density. For gram-positive species, Biolog GP profiles after growth in R2A broth and on R2A agar were also obtained.

Model communities. The model soil bacterial communities used were identical to those previously reported (3). Model bacterial communities were constructed by combining known proportions of 6 (community I-A and I-B) or 4 (community II-A and II-B) of the 10 isolates. A and B designate replicate experiments conducted in November 1992 (A) and April 1993 (B). Community I consisted of equal proportions of the following six soil bacteria: *Corynebacterium* sp. strain 5, *Pseudomonas* sp. strain 14, *Cytophaga* sp. strains 19 and 72, *Arthrobacter* sp. strain 42, and *Bacillus* sp. strain 81. Community II consisted of *Bacillus* sp. strain 94

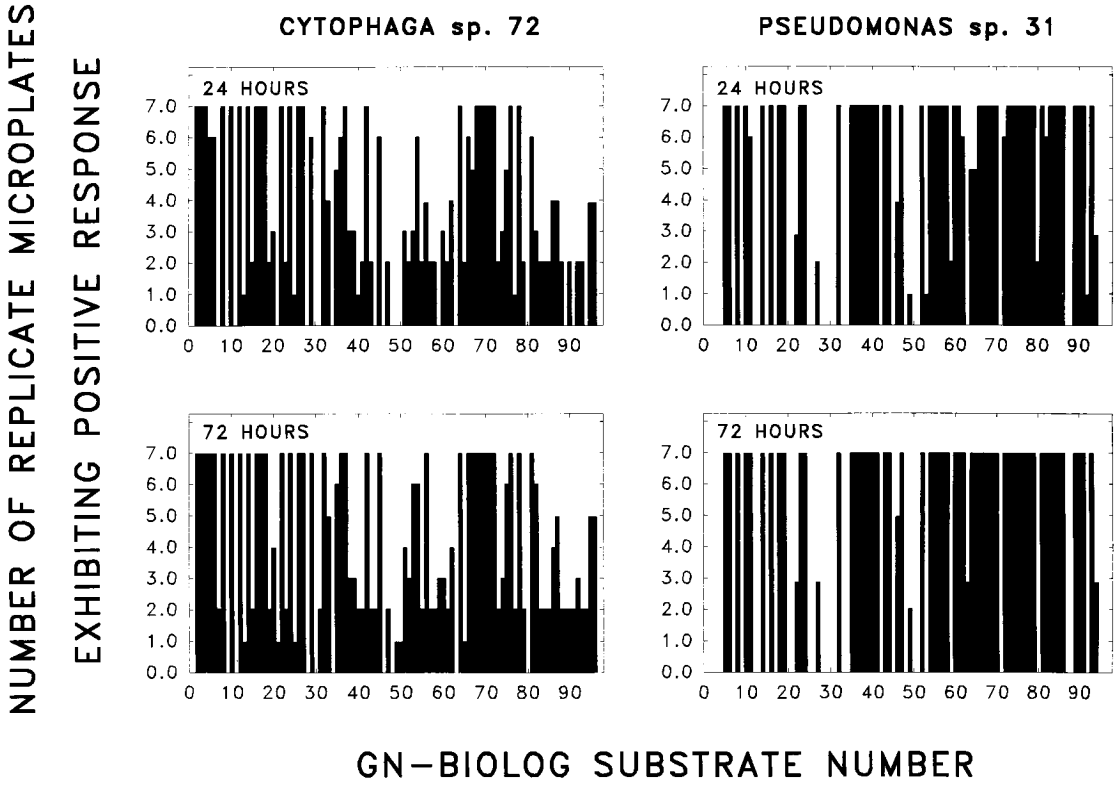

FIG. 1. Number of positive responses at 24 and 72 h, as measured by Microlog 2N software, for each of the 95 Biolog GN substrates after inoculation of seven replicate microplates with *Cytophaga* sp. strain 72 or *Pseudomonas* sp. strain 31.

(which made up half of the community on a cell number or biomass basis) and *Corynebacterium* sp. strain 5, *Pseudomonas* sp. strain 31, and *Cytophaga* sp. strain 47, each making up one-sixth of the community.

Soil communities. Soil was obtained from a greenhouse plantings of corn, at the termination of an experiment conducted by Hal Collins at the Kellogg Biological Station, Michigan State University. Soil was originally obtained from a single location at the Kellogg Biological Station Long Term Ecological Research Site. It was homogenized, dried, and placed in 10-gal (38-liter) plastic pots. Water was added to field capacity, and corn was planted in each pot 2 weeks later. In each pot, a single corn plant was allowed to reach physiological maturity, at which time soil samples (100 g) were taken from three locations within the root zone and three locations within the bulk soil from each of six pots (36 samples). Each of the 36 1-g samples of soil was suspended in 5 ml of 50 mM phosphate buffer (pH 7) and agitated for 15 min. Approximately one granule of $CaCl_2$ and an equivalent amount of $MgCO_3$ were added to each suspension, and the resulting floc was allowed to settle. This left a slightly turbid supernatant, which was directly inoculated onto Biolog MT microplates. The OD_{590} values of the inoculated microplates were read, and then the plates were allowed to sit at room temperature until ~20 h, when color development began to be visible. At this time, the microplates were placed in the Bio-Tek reader, and the OD_{590} was determined at 2-h intervals for the next 24 h. The ability of each cell suspension to utilize asparagine, aspartate, glucose, cellobiose, carboxymethylcellulose, *N*-acetylglucosamine, and ferulic acid (Sigma Chemical Co.) was determined with Biolog MT plates.

PCA. Principal-components analysis (PCA) was performed with SAS/STAT (Statistical Analysis System; SAS Institute Inc., Cary, N.C.) version 6.03 under the PRINCOMP procedure. Data for each replicate of each community in the model community experiments were entered as the percent change from the substrate blank value provided by the Biolog software. Data for experiments with MT plates were entered as the blank-adjusted OD_{590} readings obtained from the Bio-Tek software.

RESULTS

Biolog GN profiles for isolates. The pattern of positive and negative responses to the 95 substrates in Biolog GN microplates was shown to be characteristic of and generally reproducible for *Cytophaga*, *Pseudomonas*, and *Corynebacterium* strains and was similar for organisms of the same genus. Biolog GN profiles for seven replicate microplates of *Cytophaga* sp. strain 72 and *Pseudomonas* sp. strain 31 at 24 and 72 h are depicted in Fig. 1. For both strains, some substrates were always utilized and were scored positive in seven of seven replicates at 24 h, while other substrates were scored negative in every replicate, even after 72 h of incubation.

The three *Cytophaga* spp. exhibited profiles similar to that of strain 72 (Fig. 1); each consistently used relatively few (18 to 25) of the 95 substrates on GN microplates and metabolized primarily carbohydrates (substrates 6 to 33), amino acids (substrates 64 to 83), and polymers (substrates 1 to 5). The two *Pseudomonas* strains metabolized primarily carboxylic acids (substrates 36 to 59), amino acids, and various carbohydrates. Neither used the carbohydrate polymers (α-cyclodextrin, dextrin, and glycogen) used by all three *Cytophaga* spp. *Corynebacterium* strain 5 used 21 of the substrates on GN microplates (Tween 40 and 80, seven carbohydrates, four carboxylic acids, bromosuccinate, succinamic acid, two amino acids, two esters, 2,3-butanediol, and glycerol).

The *Bacillus* and *Arthrobacter* spp. failed to consistently yield positive responses on Biolog GN (or GP) microplates. After 24 or 72 h, microplates inoculated with the *Bacillus* strains would typically exhibit only one to three very weak positive responses (i.e., OD_{590} only slightly above that of the substrate blank). The substrate wells identified as positive were not consistent from assay to assay, and this pattern was often accompanied by color in the substrate blank well. Slightly different results were obtained for the two *Arthrobacter* species. These strains often produced color in the substrate blank well and occasionally exhibited a profile with clear positive and negative responses. Unfortunately, these profiles were inconsistent from assay to

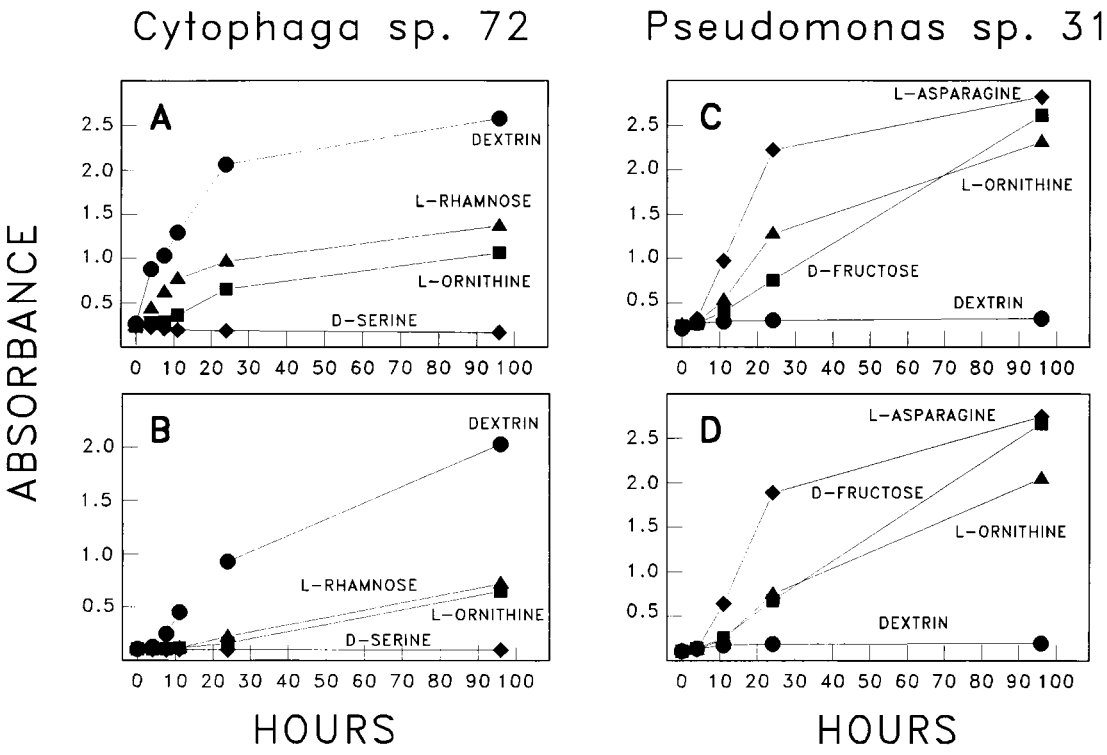

FIG. 2. Color development profiles after inoculation at two different initial cell densities (3×10^8 cells per ml [A and C] and 3×10^7 cells per ml [B and D]) for *Cytophaga* sp. strain 72 and *Pseudomonas* sp. strain 31, displaying positive, negative, and variable readings on selected Biolog GN substrates.

assay and even within replicate microplates inoculated with the same culture, so that a reference profile for these two strains could not be obtained.

The lack of substrate utilization by *Bacillus* strains was not due to tetrazolium dye toxicity (both species would grow in R2A broth with tetrazolium dye added) or to spore formation prior to inoculation of microplates. No growth medium or temperature tested resulted in a consistent Biolog response for either *Bacillus* strain. The manufacturer suggests that oligotrophic microorganisms may give an all-negative response in Biolog microplates and that color in the substrate blank well could be due to spore formation in the wells, cell lysis, or utilization of endogenous or extracellular storage polymers. To check our technique, we grew *Bacillus cereus* ATCC 14579 by recommended methods and inoculated it to Biolog GP (and GN) microplates. In contrast to our soil strains, this strain produced clearly positive results after 24 h and was identified by the Biolog software as *Bacillus thuringiensis/cereus* (similarity index, 0.51). The manufacturer also suggests that particular growth media may be required to elict a Biolog response from bacterial genera. Our experimental objectives required identical growth conditions for each strain making up our model communities; therefore, we made no effort to find alternative growth conditions that would result in consistent Biolog responses for the *Bacillus* and *Arthrobacter* isolates. We believe that our approach reflects what occurs when natural microbial communities are extracted from soil and inoculated to Biolog plates, since in situ conditions are unlikely to be optimal for stimulating a Biolog response by every community member.

Kinetics of substrate oxidation by isolates. The variable responses of *Cytophaga*, *Pseudomonas*, and *Corynebacterium* strains were shown to result from weak, nonlinear rates of substrate oxidation combined with a dependence of substrate oxidation rate on inoculum cell density. The basis for positive, negative, and variable responses can be seen in Fig. 2, which shows the time course of color development (OD_{590}) on selected substrates for either *Cytophaga* sp. strain 72 or *Pseudomonas* sp. strain 31 following inoculation at two different initial densities. Since the rate of substrate oxidation was not always linear throughout the test period, different scores could be obtained depending on when the microplate was read. For example, wells could be scored negative at early readings and positive later (e.g., fructose for *Pseudomonas* sp. strain 31) or positive at early readings but negative later (e.g., compare 10- and 96-h values for rhamnose for *Cytophaga* sp. strain 72). In addition, the rate and extent of color development depended on inoculum density (Fig. 2B and D). Therefore, assignment of a positive or negative response at 24 h for weakly oxidized substrates was inconsistent from one replicate microplate to another. In contrast to variable substrates, substrates which were always scored positive for a particular strain (for example, dextrin for *Cytophaga* sp. strain 72 and asparagine and ornithine for Pseudomonas sp. strain 31 [Fig. 2]) were oxidized rapidly and to a greater extent.

Additional experiments were done to determine the effect of inoculum density and cell growth in microplate wells on substrate oxidation kinetics. Previous results showed that *Cytophaga* sp. strain 72 always exhibited a positive response on galactose, that *Pseudomonas* sp. strain 31 always exhibited a variable response, and *Corynebacterium* sp. strain 5 would not oxidize galactose. We examined details of galactose oxidation kinetics by these three strains and measured cell growth of each strain following inoculation to Biolog microplate wells at two different inoculum densities (Fig. 3). *Cytophaga* sp. strain 72 exhibited a rapid and extensive oxidation of galactose (Fig. 3A and B) and either maintained viable cell count at 10^8 cells per ml (Fig. 3A) or grew to 10^8 cells per ml (Fig. 3B) and then maintained their cell count at this density. *Pseudomonas* sp.

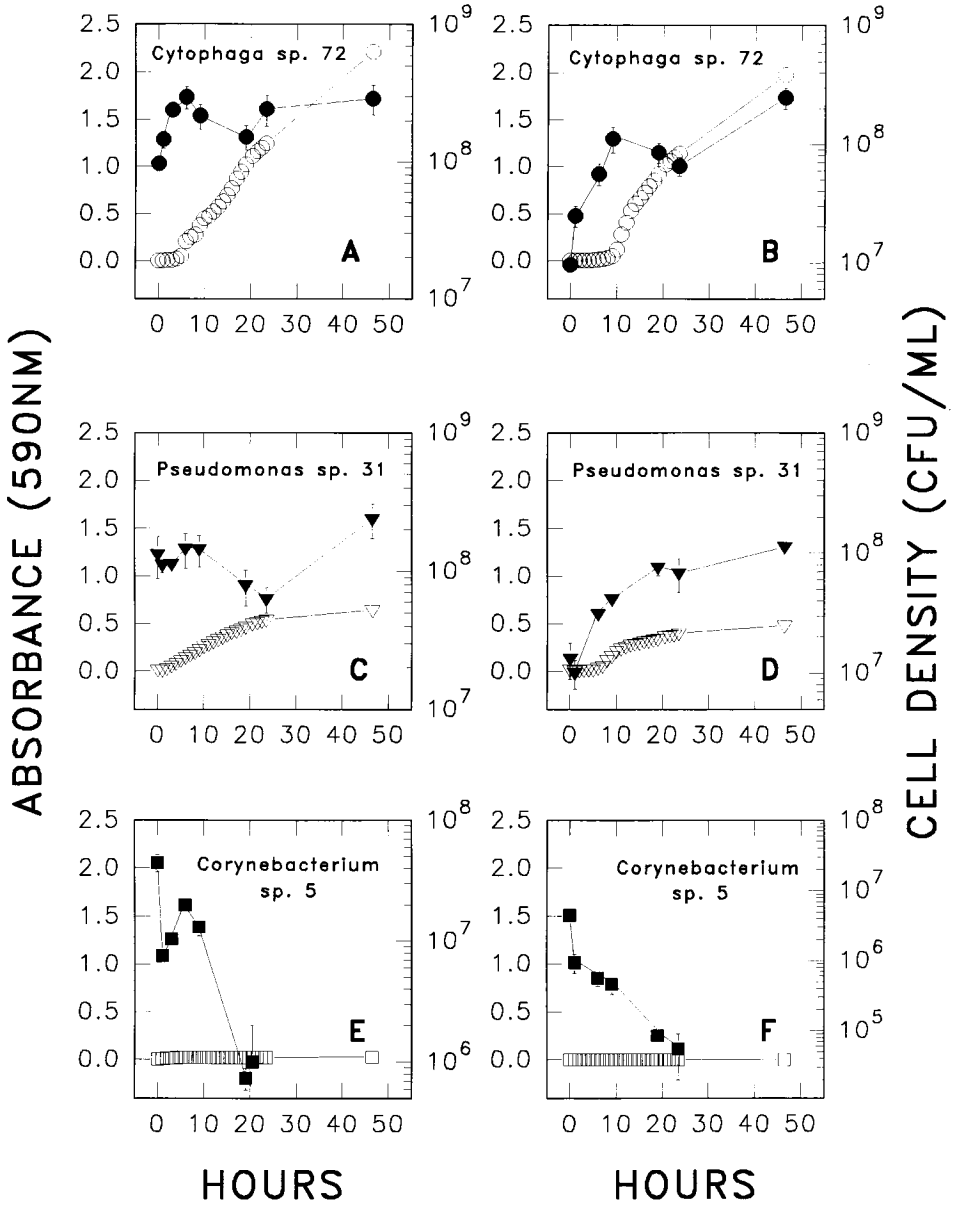

FIG. 3. Color development profiles (OD_{590}; open symbols) and cell density (CFU per milliliter on R2A agar; solid symbols) in wells of Biolog MT microplates containing 0.25% (wt/vol) galactose, following inoculation with three different bacterial strains each at two different initial concentrations. (A, C, and E) Initial inoculum density, $\sim 10^8$ CFU/ml; (B, D, and F) initial inoculum density, $\sim 10^7$ CFU/ml.

strain 31 exhibited a more moderate rate of galactose oxidation yet gave a similar pattern of viable cell counts to that of *Cytophaga* sp. strain 72. For both *Cytophaga* sp. strain 72 and *Pseudomonas* sp. strain 31, cell counts in the blank well were maintained at or increased to 10^8 CFU/ml without significant color development (data not shown). The predominant effect of inoculum dilution for *Cytophaga* sp. strain 72 and *Pseudomonas* sp. strain 31 was a lag in the initiation of color development. *Corynebacterium* sp. strain 5 did not oxidize galactose, and the viable cell count decreased throughout the course of the experiment (Fig. 3E and F). Therefore, while cell growth does occur in Biolog microplate wells, the amount of growth is not correlated to the rate or extent of substrate oxidation. The rate and extent of oxidation of particular substrates for isolates inoculated at similar densities were reproducible, however.

Environmental effects on Biolog GN profiles of isolates. Environmental conditions (growth temperature, growth medium, solid versus liquid culture) had very little affect on substrate utilization profiles by isolates. We noted only a few exceptions. *Pseudomonas* sp. strain 31 did not use formic acid after growth on R2A agar or nutrient agar, even though this substrate was always used after growth in R2A broth. *Cytophaga* sp. strain 72 used five additional substrates after growth on nutrient agar or trypic soy broth agar and used two additional substrates after growth on R2A agar, as compared with when it was grown in R2A broth. The only observed temperature effect was that *Pseudomonas* sp. strain 31 occasionally used formic acid after growth at 17°C but never at other temperatures.

GN Biolog profiles of model communities. Substrate utiliza-

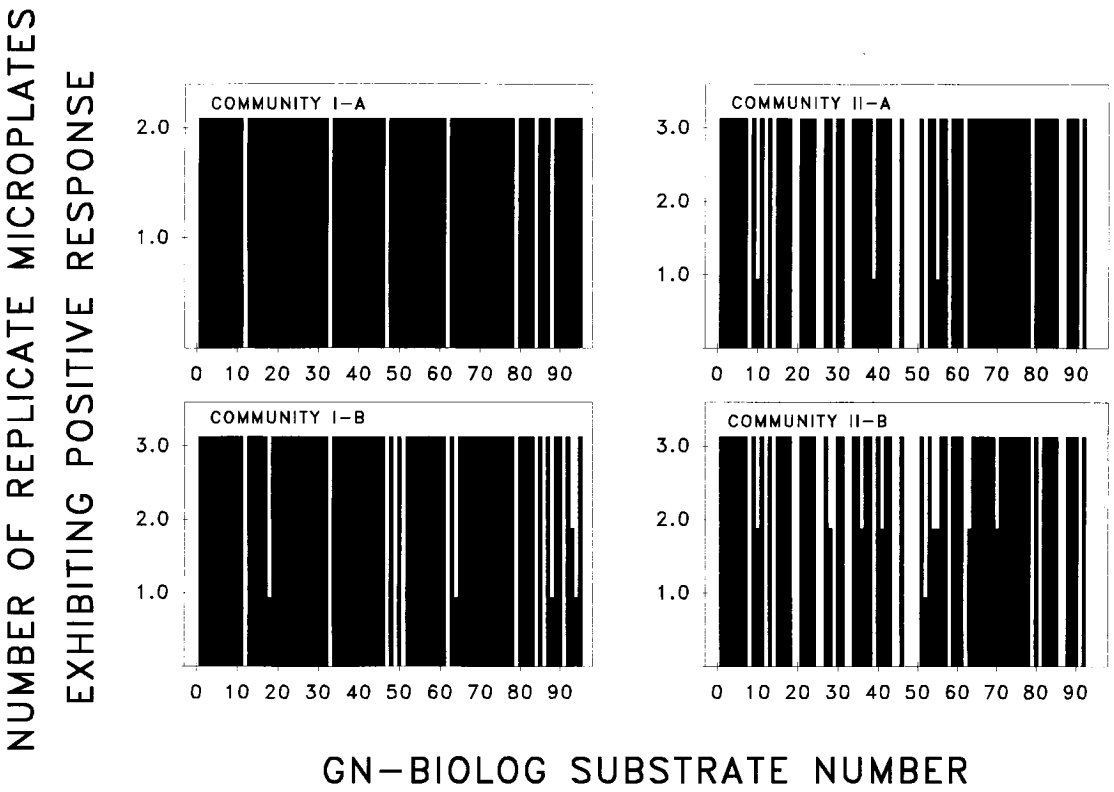

FIG. 4. Number of positive responses at 72 h, as measured by Microlog 2N software, for each of the 95 Biolog GN substrates after inoculation of replicate microplates with model bacterial communities composed of six soil bacterial isolates (community I) or four soil bacterial isolates (community II). A and B designate two independent constructions of the identical model community.

tion profiles of whole microbial communities were repeatable and unique for each community. For these tests, we used model bacterial communities composed of six (community I) or four (community II) strains. We specifically included *Bacillus* and *Arthrobacter* strains in each community, to see if these isolates would affect community profiles in any way, even though when tested as axenic cultures they failed to oxidize any substrates. Figure 4 depicts the 72-h Biolog GN profile for each community from two independent experiments. PCA clearly indicated the degree to which profiles for each community were unique and reproducible. PCA scores obtained after analysis of the covariance matrix of 72-h OD_{590} values are depicted in Fig. 5. PCA based on the covariance matrix distinguished the two model communities, and clustered replicates of each community from both experiments, on the first PC axis (Fig. 5). The first covariance PC axis accounted for 65.4%, the second accounted for 27.3% and the third accounted for 3.9% of the variation in the data, for a cummulative total of 96.6%. PC axis 1 had highest loadings on *m*-inositol, D-raffinose, α-ketoglutaric acid, hydroxy L-proline, L-ornithine, and urocanic acid (all ~0.20) and on lactulose (−0.21). These were substrates for which there were consistent differences in the degree of substrate oxidation (i.e., OD_{590} values) between the two communities. Community II replicates exhibited much higher 72-h OD_{590} values on *m*-inositol, D-raffinose, α-ketoglutaric acid, hydroxy L-proline, L-ornithine, and urocanic acid and much lower values on lactulose than did community I replicates in either experiment.

Profiles obtained for each community at earlier times (24 h) were also unique and reproducible for each community. In this case, PC axis 1 accounted for 89% of the variation and the first three PC axes accounted for 97% of the variation in the data. The first PC axis was most highly correlated with oxidation of *m*-inositol, *cis*-aconitic acid, citric acid, D-gluconic acid, α-ketoglutaric acid, D-saccharic acid, L-asparagine, and hydroxy L-proline (all ~0.26). There were no equivalent negative loadings for PC axis 1. Again, these were substrates with the highest percent change values in community II-A and II-B and rela-

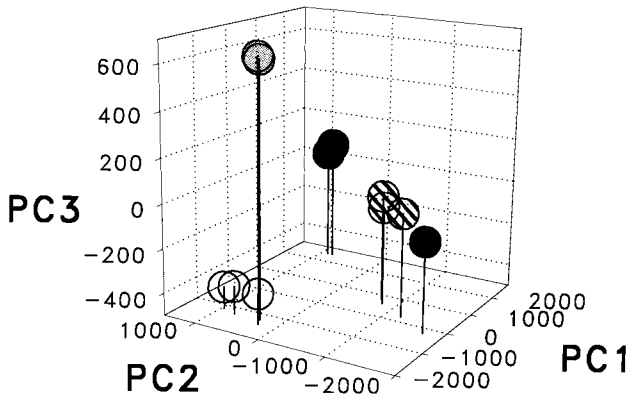

FIG. 5. Results of PCA of 72-h OD_{590} readings taken from Biolog GN microplates following inoculation with model bacterial communities composed of six soil bacterial isolates (community I) or four soil bacterial isolates (community II). A and B designate two independent constructions of the identical model community. PC1, PC2, PC3, first three principal-component axes. Symbols: ◍, community I-A; ○, community I-B; ◍, community II-A; ●, community II-B.

tively low values in Community I-A and I-B at 24 h, and the substrates include some of those important in the analysis of the 72-h data.

Effect of community composition on Biolog GN profiles. Positive responses to substrates in community profiles generally reflected the presence of specific strains capable of using those substrates. Biolog GN profiles of axenically grown strains were used to identify "signature compounds" that indicated the presence of particular strains in our model community profiles. For example, all the "signature" substrates for *Pseudomonas* sp. strain 14 or 31 (D-alanine, alaninamide, D-glucosaminic acid, L-histidine, D-galactonic acid lactone, malonic acid, or hydroxy L-proline) were positive after 72 h of incubation. Similarly, the carbohydrate polymers and glycyl-L-aspartic acid were positive in both communities at 72 h, indicating the presence of *Cytophaga* sp. strains 19 and 72 in community I-A and I-B and uniquely indicating the presence of *Cytophaga* sp. strain 47 in community II-A and II-B. In addition, β-methyl glucoside (*Cytophaga* sp. strain 72) and D-raffinose (*Cytophaga* sp. strain 47) uniquely indicated the presence of these strains in their respective communities. Only one unexplained positive reading was found. D-Raffinose (substrate 27) was scored as a positive response for every replicate of community I at 72 h, even though none of the community members used this substrate when tested alone.

Negative responses at later reading times generally indicated the absence of strains capable of using a given substrate. The only exception was that of the three substrates indicative of the presence of *Corynebacterium* sp. strain 5 in each community, xylitol (substrate 33, Fig. 4) was negative in every case following 72 h of incubation. At earlier reading times, false-negative results were more frequently noted, since signature substrates were not necessarily positive after 24 h of incubation. For example, 2,3-butanediol (substrate 91, Fig. 4) and thymidine (substrate 87), indicative of *Corynebacterium* sp. strain 5, were negative at 24 h for each community. The presence of *Arthrobacter* sp. strain 42 or *Bacillus* sp. strain 81 in community I or of *Bacillus* sp. strain 94, which made up half the total cell population of community II, appeared to have no effect on community profiles.

Effect of community composition on degree of substrate oxidation. Since PCA grouped our communities on the basis of responses which were consistently higher in one community than the other, we examined the underlying reason for relatively high responses, asking whether they might reflect the presence of several microorganisms using the same substrate or a single highly active strain. We found no correlation between the number of strains able to utilize a substrate and the degree of substrate oxidation. For example, while there were two *Cytophaga* spp. in community I but only one *Cytophaga* sp. (strain 47) in community II, there was no pattern to the responses to α-cyclodextrin, dextrin, and glycogen that would suggest greater utilization in community I than in community II. Similarly, in both community I (*Corynebacterium* sp. strain 5, *Pseudomonas* sp. strain 14, and *Cytophaga* sp. strain 19) and community II (*Corynebacterium* sp. strain 5, *Pseudomonas* sp. strain 31, and *Cytophaga* sp. strain 47), there were three strains able to oxidize *m*-inositol and yet community I consistently exhibited lower responses to *m*-inositol than community II (so much so that *m*-inositol contributed significantly to the PCA).

Kinetics of substrate oxidation by communities. Although the rate and extent of substrate oxidation by communities were reproducible community parameters, they were not simply the sum of those exhibited by the isolates that formed the community. Communities composed of three strains (*Corynebacterium* sp. strain 5, *Cytophaga* sp. strain 72, and *Pseudomonas* sp. strain 31) were constructed, and the rate of galactose oxidation by the community, as well as the viable cell count for each community member at eight times during incubation, was determined (Fig. 6A and B). The rate of galactose oxidation for the community of three isolates was intermediate between the profiles of *Cytophaga* sp. strain 72 and *Pseudomonas* sp. strain 31 (Fig. 6A and B). Neither of these strains exhibited any pattern in viable counts which would explain the profile of the community (Fig. 6B). *Corynebacterium* sp. strain 5, inoculated at a density of 6×10^7 CFU/ml, could not be detected in the wells (detection limit, $\geq 10^4$ CFU/ml) at any subsequent time in the experiment. The most pronounced effect of inoculum density was a lag in color development (data not shown). In experiments in which each of the three community members was paired with one other member (data not shown), *Corynebacterium* sp. strain 5 had no effect on the rate of substrate oxidation exhibited by either *Cytophaga* sp. strain 72 or *Pseudomonas* sp. strain 31. However, when *Cytophaga* sp. strain 72 was paired with *Pseudomonas* sp. strain 31, the resulting profile was intermediate between the profiles of the two individual strains and appeared similar to the community profile depicted in Fig. 6B. This result confirmed those obtained with our model communities in that the degree of substrate oxidation exhibited by a community does not reflect the number of organisms in the community that are able to utilize that substrate. Similar experiments were done with additional test substrates (Fig. 6C to F). Color development profiles for both isolates and combinations were substrate specific and highly reproducible (Fig. 6).

Soil communities. On the basis of the results obtained with model communities, we anticipated that whole-community substrate utilization patterns could be used to distinguish between rhizosphere and bulk soil communities. In experiments with soil microbial community samples taken from greenhouse plantings, we were unable to group such communities on the basis of the presence or absence of a positive response to specific substrates or their kinetics of substrate oxidation. The lack of similarity among sample replicates suggested microscale spatial heterogeneity in these soils. Figure 7 depicts these results and shows that (i) there was no pattern of substrate utilization or kinetic profile distinguishing rhizosphere from bulk soil, either within or between pots, and (ii) there was no reproducible kinetic profile, especially on cellobiose or *N*-acetylglucosamine, within rhizosphere or bulk soil samples from the same pot. Patterns were equally variable for all other samples. Although we did not determine the inoculum density for each soil suspension, one tested rhizosphere sample (pot 1, Rhiz 1, Fig. 7) contained 7.5×10^5 CFU/ml at the time of inoculation and exhibited values ranging from 1.25×10^8 to 9×10^8 CFU/ml in every well, including the blank, at the time color development began (20 h after inoculation). There was no significant color development in the blank well associated with this increase in cell density. All samples initiated color development at about the same time (Fig. 7), which suggests equivalent inocula. Readings (OD_{590}) of all 36 samples at 30 h (approximate midpoint of color development profile) were analyzed by PCA, but no separation of samples with regard to origin (pot number) or type of sample (rhizosphere versus bulk soil) was possible.

DISCUSSION

Community-level carbon source utilization (Biolog) profiles have recently been introduced as a means of classifying microbial communities on the basis of heterotrophic metabolism (2). Such a classification system might allow microbial ecologists to

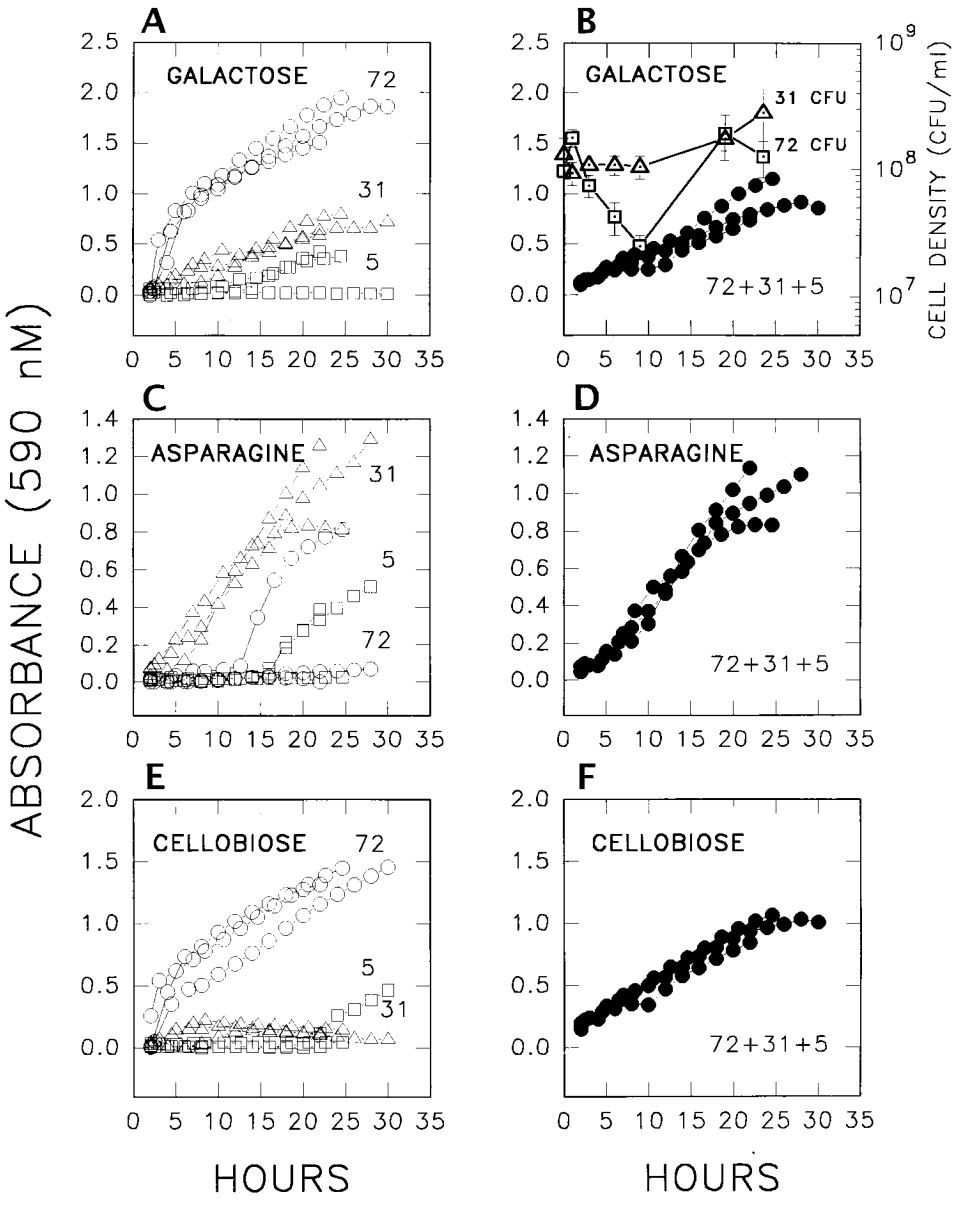

FIG. 6. Triplicate color development profiles (OD_{590}) exhibited by three bacterial strains (72, *Cytophaga* sp. strain 72; 31, *Pseudomonas* sp. strain 31; 5, *Corynebacterium* sp. strain 5) inoculated independently (A, C, and E) or as a combination of all three strains (B, D, and F) to wells of Biolog MT microplates containing 0.25% (wt/vol) galactose (A and B), asparagine (C and D), or cellobiose (E and F). Panel B (open symbols) also indicates the cell density of *Cytophaga* sp. strain 72 and *Pseudomonas* sp. strain 31 at selected intervals during one experiment. *Corynebacterium* sp. strain 5, inoculated at a cell density of $\sim 6 \times 10^7$ cells per ml, was not detected at any of the indicated intervals during the same experiment.

compare the metabolic roles of microbial communities from different environments without involving tedious isolation and identification of community members. The simplicity of the method and the commercial availability of Biolog plates are particularly attractive. However, no tests of the reproducibility of community Biolog profiles or of their accuracy in depicting community structure or activity have been reported. Our use of model soil bacterial communities for which the taxonomic structure and expected carbon substrate utilization pattern were known a priori allowed us to determine the strengths and weaknesses of this approach to microbial community classification.

Our experiments confirm that more-dilute inocula result in protracted rates of color development for both isolates and simple communities. Both Garland and Mills (2) and Winding (5) noted a correlation between inoculum cell density and the rate of color development and dealt with this problem in different ways. Winding (5) and Zak et al. (6) recorded data after 3 days and after 60 h of microplate incubation, respectively, and neither made adjustments for different inoculum cell densities. Garland and Mills (2) adjusted for this phenomenon by calculating an average well color development value for each microplate. This value was obtained by calculating the raw difference between the optical density in each well and that in the control well and then summing all these values and dividing by 95. Every value for each substrate was then divided by the average well color development for that microplate to normalize comparisons between samples with different inoculum den-

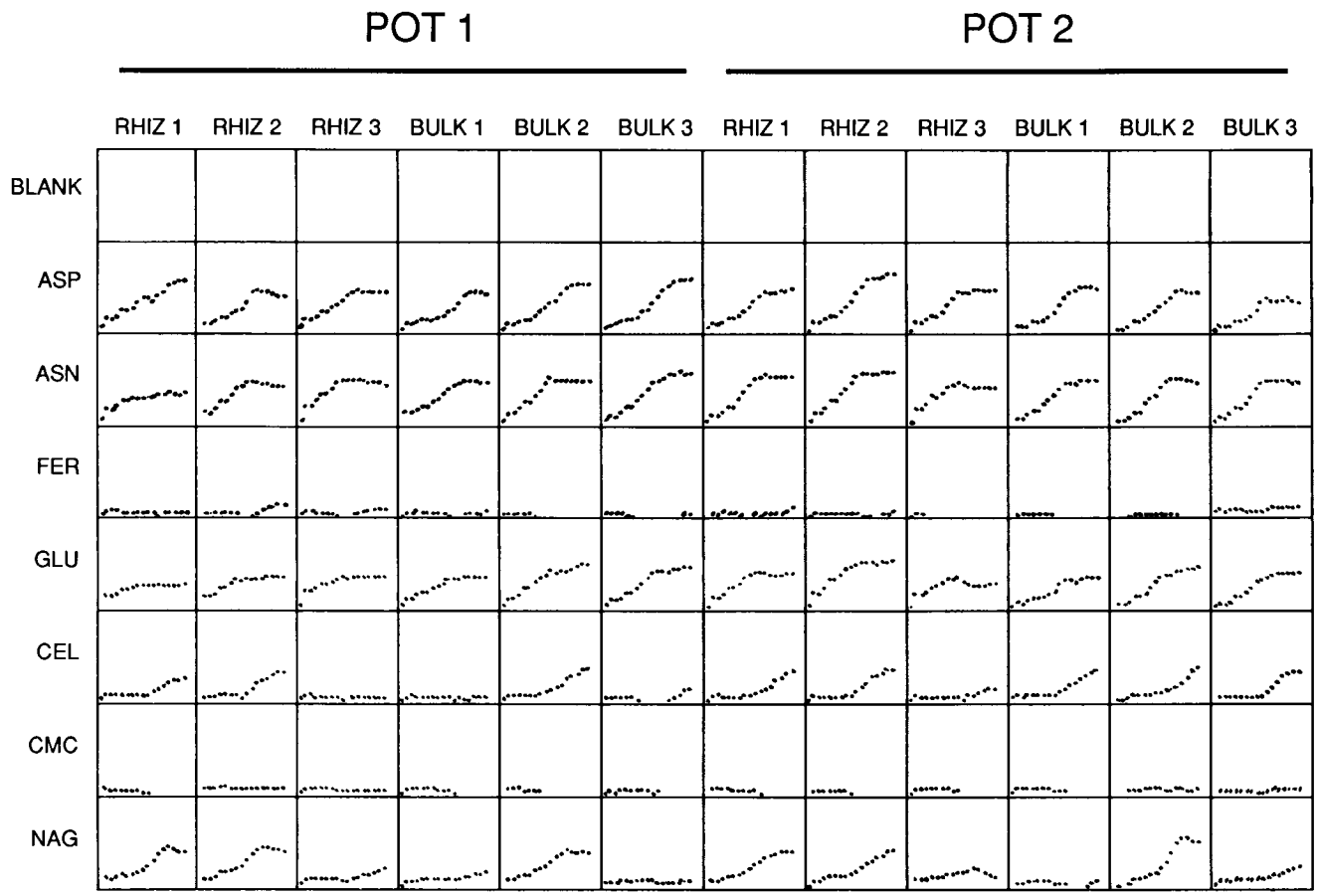

FIG. 7. Color development profiles (OD_{590}) for soil samples taken from the rhizosphere and bulk soil of greenhouse-maintained plantings of corn. Rhizosphere samples (RHIZ 1 to RHIZ 3) and bulk soil samples (BULK 1 to BULK 3) from two different pots (POT 1 or POT 2) were inoculated to a Biolog MT microplate containing seven carbon substrates. ASP, aspartate; ASN, asparagine; FER, ferulic acid; GLU, glucose; CEL, cellobiose; CMC, carboxymethyl cellulose; NAG, *N*-acetylglucosamine. Blank wells contained only buffer. Absorbance readings were initiated at 20 h postinoculation and were taken at 2-h intervals for 24 h.

sities. Our results support the conclusion of Garland and Mills (2) that optimal use of this approach requires samples of approximately equivalent inoculum densities. If this criterion is not met, community comparisons based on either (i) positive and negative responses to the 95 Biolog substrates or (ii) the degree of substrate oxidation of particular substrates will be compromised by the slow response of dilute inocula. For example, Zak et al. (6) compared the total number of substrates used and the "activity levels" (summation of absorbance values) for six different desert soil microbial communities at 12-h intervals over a 72-h incubation. One group of three communities consistently used more substrates and showed higher activity levels than the remaining three communities at every reading time, strongly suggesting an inoculum density effect. Although these authors noted some community differences in substrate utilization among the three "active" communities, the remaining (less-active) communities were grouped similarly by every statistical approach. Unfortunately, Zak et al. (6) did not report inoculum density for their samples, so the significance of their findings is not clear.

Both Garland and Mills (2) and Winding (5) noted that growth occurred in Biolog microplate wells, and both suggested that the Biolog community substrate utilization assay reflects the growth of a proportion of the community which is capable of utilizing Biolog substrates. We determined that while growth in wells did occur, especially when dilute inocula were used (Fig. 3), this growth was not correlated with the rate or extent of substrate oxidation. In our experiments, growth without attendant color development occurred in blank wells for isolates, model communities, and soil samples, and isolates displayed equivalent growth but different color development profiles on the same substrates. In simple model communities, substrate oxidation profiles were not directly correlated with the growth of isolates which used the substrate (Fig. 6).

Although substrate oxidation profiles are not a direct representation of bacterial growth, we note that Biolog substrate oxidation responses for both isolates and communities often exhibit a lag phase, an exponential phase, and a stationary phase, as do bacterial growth curves. This nonlinearity has important implications for the interpretation of single fixed-time readings of community Biolog responses, as Garland and Mills (2) first noted. Substrates extensively used by a single community after, for example, a 72-h incubation, may not be the same as those used most extensively at prior reading times. For example, in PCA of our model communities, there were only three substrates with high correlations on the first PCA axis at both 24 and 72 h. Several other substrates were significant to the PCA at only one of the reading times; consequently, the substrates interpreted by multivariate analysis to be most significant in distinguishing the communities changed over the course of the experiment. The optimum approach to the use of Biolog microplates for community analysis would

seem to require careful monitoring of color development so that effects of nonlinearity can be accounted for in the final interpretation of community response.

We have demonstrated for the first time that the pattern of positive and negative responses and substrate oxidation rate and extent were highly reproducible for simple microbial communities (Fig. 6), if inoculum density was controlled. Replicate microplates inoculated with the same microbial community and plates inoculated with two independent constructions of the identical model communities exhibited nearly equivalent Biolog GN profiles (Fig. 4 and 5). In these experiments, the microplates were inoculated with communities having similar cell densities. With the potential complication of different inoculum cell density controlled in our experiments, we obtained similar results with 24- and 72-h readings. PCA reflected the reproducibility and uniqueness of the model community profiles. Replicate microplates inoculated with the same microbial community tended to cluster on the first PCA axis, which typically accounted for >60% of the variation in the data set. Furthermore, the two different communities were clearly separated on the first PCA axis.

Our results imply that if different samples of similar inoculum density exhibit differences in the pattern of positive and negative responses to the 95 substrates in Biolog GN microplates, this probably reflects real differences in community composition. Moreover, positive and negative community responses to particular Biolog substrates may be interpreted with regard to whether utilizers of those substrates are present in the community. Our experiments do not address the impact of rare community members on community substrate utilization profiles. However, we would anticipate that dramatic differences in response to a substrate across replicates (e.g., negative in most replicates but strongly positive in one) might indicate the presence of rare community members in the inoculum.

If communities exhibit differences in the rate and extent of oxidation of particular Biolog substrates and these communities are segregated in multivariate analyses on the basis of differences in the degree of oxidation of particular substrates, does this indicate, as suggested by others (2, 5, 6), real differences in community function or metabolic potential? Our results suggest that interpretation of substrate utilization profiles with regard to either community taxonomic composition or numbers and activities of substrate utilizers will be complicated for two reasons. First, the failure of some bacterial strains to produce any response in Biolog microplates suggests that the microplate environment is "selective" and therefore will de facto fail to represent the metabolic activity of all members when inoculated with whole-community samples. Bacteria which produce no response in Biolog microplates may do so because the cells fail to maintain viability (as noted for the negative response of *Corynebacterium* sp. strain 5 to galactose in Fig. 3) or because they do not use the substrate. The presence of *Corynebacterium* sp. strain 5 in combination with *Cytophaga* sp. strain 72 and *Pseudomonas* sp. strain 31 had no effect on their rates of substrate oxidation, and *Bacillus* strains, which gave no response in Biolog GN plates, had no effect on the community responses to all 95 substrates in Biolog GN microplates. On the basis of these results, we assume that the presence of nonactive bacteria in a community may have no detectable effect on the community profile. This phenomenon will complicate any attempt to extrapolate from community Biolog profiles to in situ community activities.

Second, the kinetic profile for a combination of isolates is not a simple summation of their individual profiles; therefore, the degree of oxidation of particular substrates cannot be interpreted with regard to the number or activity of community members carrying out that oxidation. While kinetic profiles for isolates and communities were highly reproducible, there was no means to predict isolate or community responses. For communities of two or three isolates, we found kinetic patterns in which the rate and extent of color development were less than or equal to those of the most active member. Importantly, we noted no additive community response, and there was no evidence for synergism between isolates in the kinetic analyses. This means that the degree of substrate oxidation is not a function of the number of utilizers or of their individual activities. This conclusion was further substantiated by our experiments with model communities, for which we found no correlation between the number of utilizers and the degree of substrate oxidation. In fact, for some substrates which were significant in the differentiation of the two communities by PCA, there were the same number of utilizers in both communities.

In general, PCA separated our two model communities on axes which could be interpreted in logical and expected ways, typically on the basis of responses which were very high in one community and correspondingly low or negative in the other community. The emphasis on negative results is important, since our data indicate that nonutilization of a substrate by a community accurately reflects the likelihood that no, or rare, community members in the inoculated sample would produce a Biolog response on that substrate. The dependence of PCA separation on degree of substrate oxidation is more problematic because (i) the degree of substrate oxidation at a particular time may be a function of inoculum cell density, (ii) different microorganisms which use the same substrates oxidize those substrates to different extents, and (iii) the substrate oxidation profiles of communities are not simple summations of the individual profiles of their constituent members. Environmental samples may contain several populations capable of utilizing a given substrate, all at undetermined cell densities, and exhibiting a variety of kinetic responses in Biolog microplates. Our research with isolates and simple combinations of isolates clearly indicates that while whole-community substrate utilization profiles constitute reproducible signatures useful in classifying microbial communities, differences in the rate and extent of color development associated with such samples cannot be interpreted with regard to the number of utilizers or to the metabolic potential or function of the community.

We found that the patterns and kinetics of substrate utilization varied between replicate samples of rhizosphere or bulk soil. This in itself may be significant, since we have clearly demonstrated that both within-well substrate oxidation rates and the pattern of responses to multiple substrates are highly reproducible for both isolates and communities. We have also shown that differences in community kinetic profiles on a given substrate are most probably due to differences in community composition if inoculum density is not a factor. As a result, differences in substrate utilization profiles for replicate samples from soil may indicate real differences in community composition. If this is the case, whole-community substrate utilization profiles might be used to assess the fine structure of microbial community distribution across relatively small environmental gradients. Winding (5) reported the ability to discriminate between different size fractions of the same soil by using Biolog GN profiles, but since not all her samples were subjected to the same dilution, there were different initial densities of bacteria in the soil samples, and only a single reading was taken after 3 days of incubation, it was difficult to determine the basis for the observed differences.

In our experiments, differences between the bulk-soil and rhizosphere communities might have been elucidated by using

an extended suite of compounds or Biolog GN microtiter plates. In experiments not described here, we found that 42 substrates were sufficient to distinguish between our model soil communities and 21 substrates were sufficient to distinguish between experimentally manipulated stream biofilm communities (4). Therefore, the number of substrates is not as important as whether there are differences in their use by communities. We foresee that the use of researcher-selected compounds and the addition of kinetic readings will increase the potential applications and strengthen the interpretation of whole-community substrate utilization patterns. Profiles such as those shown in Fig. 7 can then be obtained to provide information useful in choosing the appropriate time to compare readings between samples and allow the investigator to assess whether features such as increased lag time associated with decreased inoculum density should be considered. Kinetic profiles can be characterized by fitting the data to an appropriate equation, and the resulting parameters determined from curve fitting could be entered into a statistical analysis, providing greater analytical power for ecological studies.

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