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ENVIRONMENTAL BIOTECHNOLOGY

Patrick D. Schloss · Anthony G. Hay · David B. Wilson · James M. Gossett · Larry P. Walker

Quantifying bacterial population dynamics in compost using 16S rRNA gene probes

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Abstract Composting provides a dynamic setting for studying ecological topics such as succession, competition, and community stability in a relatively short period of time. This study used hierarchical small sub-unit-based rRNA gene probes to quantify the change in the relative abundance of phylogenetic groups common to compost in laboratory scale reactors. Bacterial 16S rRNA gene targets accounted for only 37% of all small subunit (SSU) rRNA genes initially, but increased to a maximum of 83% of the total at 84 h. The sum of rRNA genes detected using probes specific to *Pseudomonas* and low-G+C Gram-positive rRNA genes represented between 16% and 87% of the total. The lack of hybridization to the taxon-specific probes was most pronounced between 36 h and 60 h, when the pH was between 4.6 and 4.8. During this period the relative abundance of taxon-specific gene targets accounted for only 17–33% of the total bacterial rRNA gene targets. *Pseudomonas*-type 16S rRNA genes were the most abundant of the groups measured until 72 h. Those genes had their highest relative abundance at 12 h (78% of bacterial rRNA genes; 30% of all rRNA genes), after which time their relative abundance began to decline as the temperature increased. Prior to 72 h, 16S rRNA genes from low-G+C Gram-positive bacteria (LGC-GPB) represented less than 7% of the bacterial rRNA genes. However, by 84 h the relative abundance of LGC-GPB and *Bacillus* rRNA genes had increased to 60% and 18% of the bacterial rRNA gene targets, respectively (50% and 15% of all rRNA genes, respectively).

P. D. Schloss · L. P. Walker (✉)
Department of Biological and Environmental Engineering,
Riley-Robb Hall, Cornell University,
Ithaca, NY, 14853, USA
e-mail: lpw1@cornell.edu
Fax: +1-607-2554080

Present address:
P. D. Schloss
Department of Plant Pathology, University of Wisconsin-Madison,
Madison, WI, USA

A. G. Hay
Department of Microbiology, Cornell University,
Ithaca, NY, 14853, USA

D. B. Wilson
Department of Molecular Biology & Genetics, Cornell University,
Ithaca, NY, 14853, USA

J. M. Gossett
School of Civil and Environmental Engineering, Cornell University,
Ithaca, NY, 14853, USA

Introduction

Composting is generally an aerobic degradation process that is driven by microbial populations growing on a complex organic substrate. Byproducts of this process include heat, organic acids, carbon dioxide, ammonia, and biomass (Haug 1993). Changes in these byproducts can have differential effects on microbial populations resulting in a complex feedback system between abiotic and biotic factors in the substrate. Due to difficulties in tracking and quantifying the relationships between physical factors and population sizes, it has not been possible to fully understand the ecological processes important in composting. Identifying these relationships has been complicated by the presence of an uncontrolled bacterial inoculum that varies between operations due to variations in material handling, air conditioning, and substrate composition (Schloss et al. 2003b). Although several previous composting studies have attempted to quantify bacterial populations using plating techniques (Nakasaki et al. 1985; Strom 1985a), we are not aware of any quantitative culture-independent analysis of compost bacterial community composition.

Several qualitative studies have taken snapshots of the microbial community during different phases of composting using PCR-based techniques such as fingerprint analysis (Ishii et al. 2000; Pedro et al. 2001; Peters et al. 2000; Schloss et al. 2003a,b) and/or sequencing of clone libraries (Blanc et al. 1999; Dees and Ghiorse 2001; Malik

et al. 1994). In our earlier study (Schloss et al. 2003a), identification of the most common operational taxonomic units (OTUs) from automated rRNA intergenic spacer amplification (ARISA) fingerprints showed that lactic acid bacteria (LAB) were commonly recovered during the acidic phase. The number of mesophilic and thermophilic *Bacillus*-type OTUs increased after 72 h when the pH increased. OTUs related to *Pseudomonas* sp. were identified throughout the process both in our study and in others (Ishii et al. 2000; Pedro et al. 2001; Peters et al. 2000; Schloss et al. 2003a).

Although limited by the number of phylogenetic groups that can be quantified simultaneously, nucleic acid-based hybridization techniques using DNA oligonucleotides specific to regions of 16S and 18S rRNA molecules have been successfully used to describe the changes in the microbial populations of other dynamic environmental processes (Edgcomb et al. 1999; Raskin et al. 1994; Stahl et al. 1988). Since levels of rRNA can be correlated to activity, rRNA is an ideal target in sedentary environments. However, DNA can serve the same function in more dynamic processes where there are rapid changes in bacterial populations (Edgcomb et al. 1999). In addition, rRNA is difficult to extract from a heterogeneous matrix, such as compost. In this study, we used oligonucleotide probes specific for small sub-unit rRNA genes to quantify changes in the relative abundance of phylogenetic groups in genomic DNA extracted from compost during the first 96 h.

Methods and materials

Sample collection

Composting experiments were performed in 500-ml aerated reactors using 240 g (wet basis) of a synthetic food waste (SFW). The SFW was roughly a 1:1 mix (dry basis) of dog food and wood chips and had a carbon: nitrogen ratio of 18:1. The final moisture content was 55%. No inoculum was added to the substrate. Temperature and O_2 and CO_2 concentrations were monitored with a computerized data-acquisition system described elsewhere (VanderGheynst et al. 1997b), while moisture content and pH were measured off-line by standard techniques (Schloss et al. 2003b). Details of the reactor design, data-acquisition system, and substrate preparation have been presented previously (Schloss et al. 2003b).

To develop a time course for the first 96 h of the process, three replicate reactors were sacrificed after 12, 24, 36, 48, 60, 72, 84, and 96 h. Since it was not possible to run 24 reactors simultaneously, reactors run for a single time point were performed simultaneously and each time point was performed separately during the fall of 2000 as described elsewhere (Schloss et al. 2003b).

DNA extraction and purification

DNA was extracted using a bead beating procedure that was previously shown to be highly effective at lysing bacterial cells (Howeler et al. 2003; Schloss et al. 2003a). Three 5-g compost samples were taken from each reactor and from the initial substrate mixture. Samples were mixed with 5 g glass beads (170–180-μm diameter), 10 ml TE buffer (10 mM Tris-Cl, pH 8.0; 1 mM EDTA, pH 8.0), and 500 μl 10% sodium dodecyl sulfate (SDS) in 50-ml conical centrifuge tubes. The mixture was incubated at 70°C for 60 min with periodic mixing and then vortexed twice for 2 min. The samples were centrifuged and the supernatant was removed by washing the compost debris twice with TE buffer. The DNA was purified by CTAB (hexadecyltrimethyl ammonium bromide) extraction and then precipitated using polyethylene glycol (Ausubel et al. 1987). The resulting DNA pellet was resuspended in 1 ml TE and the remaining humic acids were removed by using a commercial glass milk kit (Express Matrix, Bio101, Vista, Calif.). Purification using the glass milk followed the manufacturer's protocol and samples were resuspended in ddH_2O. This extraction and purification procedure was performed in triplicate for the finished compost and initial substrate material of each reactor. The DNA from the three extractions for each reactor was pooled for subsequent analysis to reduce intra-sample variability and to increase the amount of DNA that could be blotted onto the membranes. The DNA purification procedure yielded approximately 700 ng DNA/g wet compost.

Probe synthesis

To provide at least 10-fold excess coverage for all samples, 15 pmol probe was 5′-end labeled with [γ-^{33}P] ATP for each of the five probes listed in Table 1. These probes were selected because our previous fingerprinting and sequencing study (Schloss et al. 2003a) and studies from others (Blanc et al. 1999; Dees and Ghiorse 2001; Ishii et al. 2000; Pedro et al. 2001; Peters et al. 2000) suggested that the groups targeted by the probes accounted for most of the microbial diversity in samples from the first 96 h of composting. Labeling reactions contained 15 pmol probe, 15 pmol [γ-^{33}P] ATP (3,000 Ci/mmol, Perkin-Elmer, Boston, Mass.), 5 units T4 polynucleotide kinase (Promega, Madison, Wis.), and 10 μl kinase 10× buffer. The reaction mixture was incubated at 37°C for 1 h. Excess [γ-^{33}P] ATP was removed with a nucleotide removal kit (Qiagen, Valencia, Calif.).

Membrane preparation and hybridization

For each of the samples, 100 μl purified DNA (on average, approximately 1.5 μg community DNA) were combined with 5 μl 10 *N* NaOH in 200-μl thin-wall PCR strip-tubes (LPS, Rochester, N.Y.). The samples were then boiled in a

Table 1 Oligonucleotide probes

Probe name[a]	Sequence (5′–3′)	Target group	Standard curve organism	T_w (°C)	Reference
S-*-Univ-1392-a-A-15	ACG GGC GGT GTG TRC	Universal	*Bacillus subtilis*	46	Stahl et al. 1988
S-D-Bact-0338-a-A-18	GCT GCC TCC CGT AGG AGT	Bacteria	*B. subtilis*	55	Amann et al. 1990
S-Sc-LGC-0354-a-A-18	TGG AAG ATT CCC TAC TGC	LGC[b]-*Weissella*, *Lactobacillus*	*Lb. plantarum*	55	Meier et al. 1999[c]
S-Sc-LGC-0354-b-A-18	CGG AAG ATT CCC TAC TGC	LGC-*Bacillus*	*B. subtilis*		
S-Sc-LGC-0354-c-A-18	CCG AAG ATT CCC TAC TGC	LGC-*Lactococcus*	*L. lactis*		
S-G-Bacil-0597-a-S-22	GGG TCA TTG GAA ACT GGG GAA C	*Bacillus* sp.	*B. subtilis*	55	DuTeau et al. 1998
S-S-P.put-0597-a-S-20	ACT GCA TCC AAA ACT GGC AA	*Pseudomonas putida*	*P. putida*	55	DuTeau et al. 1998

[a]Naming convention of Alm et al. (1996)
[b]Low-G+C-containing Gram-positive bacteria
[c]It is not possible to resolve these three sub-groups of bacteria with individual probes; an equimolar mixture of three probes was used to quantify presence of LGC bacteria (Meier et al. 1999)

thermalcycler for 10 min (PTC-100, MJ Research, Waltham, Mass.) to denature the community DNA.

In accordance with the manufacturer's instructions, each replicate of denatured community DNA was blotted onto Zeta-Probe nylon membranes with a Bio-Dot apparatus (Bio-Rad, Hercules, Calif.). Five sets of membranes were produced, each for hybridization to a separate probe. After the DNA was blotted, the membranes were baked for 2 h at 80°C.

Controls and mass standards included rRNA gene-fragments that had been PCR amplified and cloned from *Bacillus subtilis*, *Lactobacillus plantarum*, *Lactococcus lactis*, *Streptococcus lividens*, *Pseudomonas putida*, *Methanosarcina barkeri*, and *Cryptococcus curvatus* (a yeast) genomic DNA. Positive and negative control spots contained 5 ng DNA. In addition, a standard curve for each probe was blotted onto the membranes to correct for variability in the specific activity of the probes. These controls and standards were blotted onto separate membranes from the samples but all membranes were hybridized with the same probe and washed together.

The membranes for each probe were pre-hybridized together in 300-ml hybridization tubes (Labnet International, Woodbridge, N.J.) with 15 ml hybridization buffer (0.5 M NaH_2PO_4, 1 mM EDTA, 7% SDS, pH 7.2) for at least 4 h (Applegate et al. 1995). Probe was then added and the membranes were hybridized overnight. The membranes were then washed twice for 1 h in 80 ml 1× SSC (0.15 M NaCl, 0.015 M sodium citrate, 1% SDS) (Stahl et al. 1988) at the appropriate T_w. Membranes were then wrapped in plastic wrap and exposed to a storage phosphor screen (Molecular Dynamics, Sunnyvale, Calif.) for at least 48 h. The signal intensity for each spot was quantified with a Storm imaging device (Molecular Dynamics) with 100-μm resolution, and compared to the appropriate standards. As a uniform set of standards was used for each probe, signal was normalized to the appropriate standard(s) rather than to oligonucleotide labeling efficiency (Fig. 1).

Results

Physical process description

The first 96 h of the process revealed dynamic changes in physical parameters and the relative abundance of the measured populations. The temperature and effluent CO_2 concentration profiles mirrored each other during the first 96 h (Fig. 2a). Between 48 h and 96 h, the temperature varied between 40°C and 50°C. The pH was initially 5.6 and dropped to 4.6 at 36 h. Between 60 h and 72 h, the pH increased from 4.7 to 8.1 (Fig. 2b). The effluent O_2 concentration remained above 20% for the first 36 h and reached its lowest level of 17.2% at 72 h before increasing to 19.3% at 96 h (Fig. 2b). The substrate did not lose an appreciable amount of moisture during the first 96 h (data not shown).

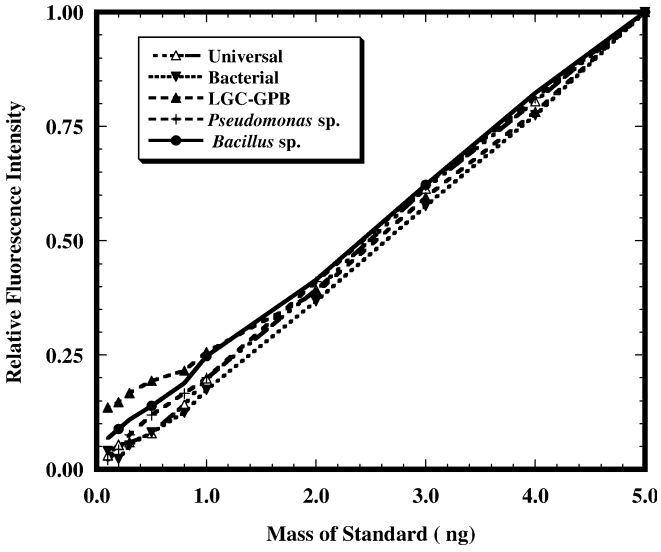

Fig. 1 Standard curves for each of the probes used in this study. Each of the probes had R^2-values above 0.99

Microbial community change

Comparison of the DNA hybridization signals from the universal and bacterial probes revealed that bacteria initially account for only 37% of total rRNA genes detected using the universal probe. The relative abundance of bacteria generally increased throughout the process, reaching a maximum of 83% after 84 h (Fig. 3). The increase in the relative abundance of total bacterial rRNA genes generally mirrored the increase in temperature during the composting process (Figs. 2a, 3).

The probe that targeted *Pseudomonas*-type organisms yielded the strongest hybridization signal during the first 60 h (Fig. 3). At 12 h, *Pseudomonas*-type rRNA genes represented 30% of the total rRNA genes but were not significantly different from the relative abundance of bacterial rRNA genes ($P>0.05$) indicating that *Pseudomonas*-type rRNA genes accounted for nearly all of the bacterial 16S rRNA genes. After 12 h, the mean relative abundance of *Pseudomonas*-type rRNA genes began to decline as the temperature began to increase and the pH began to drop. However, the 95% confidence interval for the relative abundance of *Pseudomonas*-type rRNA overlapped for each time point during this time indicating that there was not a statistically significant change in the relative abundance of *Pseudomonas*-type populations.

During the first 48 h, low-G+C-containing Gram-positive bacteria (LGC-GPB) populations were detected only at 24 h when the pH began to drop and the temperature began to increase (Figs. 2, 3). Between 60 h and 84 h, when the pH increased from 4.7 to 8.1 (Fig. 2b), the relative abundance of LGC-GPB-type rRNA genes increased from 0% to 50% of the total rRNA genes, accounting for almost all of the bacterial 16S rRNA genes as there was no significant difference from the relative abundance of bacterial rRNA genes ($P>0.05$). This increase in LAB rRNA genes coincided with a decrease in temperature from 50°C to 40°C (Fig. 2a).

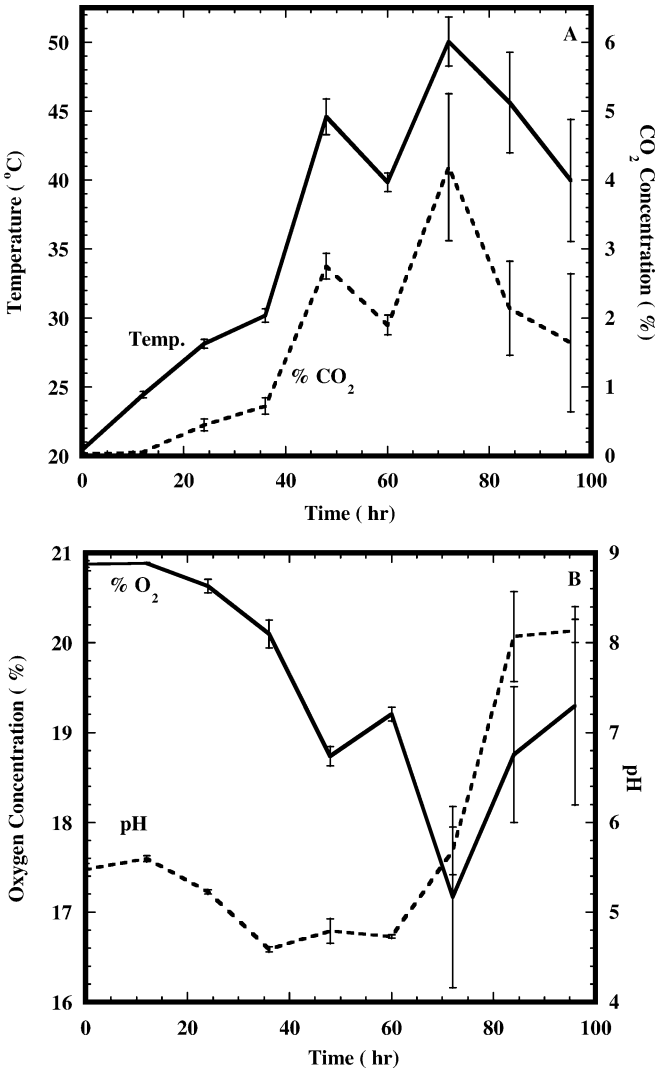

Fig. 2 **a** The mean temperature and effluent CO_2 concentration and **b** pH and effluent O_2 concentration for compost reactors operated between 0 h and 96 h. Error bars represent the 95% confidence interval of three simultaneously operated replicate reactors

Bacillus-type rRNA genes could not be detected during the first 60 h, except at 48 h. Between 60 h and 96 h, however, the relative abundance of *Bacillus*-type rRNA genes increased from 0% to 17% of universal rRNA genes (24% bacterial). The increase in the relative abundance of *Bacillus*-type rRNA genes correlated with the increase in pH and the increase in the relative abundance of LGC-GPB rRNA genes.

Discussion

The results from this study suggest that the current level of knowledge regarding the type and abundance of bacteria present during the early phases of composting is incomplete. The first evidence of this is the ability to assign only 22% of the bacterial rRNA, on average, to the targeted bacterial taxa during the acidic phase between 36 h and 60 h. This contrasts with the results obtained

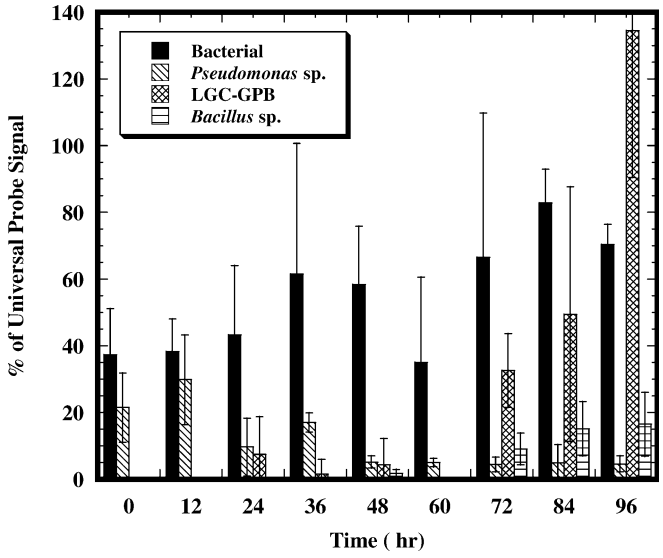

Fig. 3 The mean relative abundance of bacterial, *Pseudomonas*, low-G+C-containing Gram-positive bacteria (LGC-GPB), and *Bacillus*-type rRNA genes as a percentage of all rRNA genes detected using universal probe extracted from compost reactors operated between 0 h and 96 h. Error bars represent the 95% confidence interval of three simultaneously operated replicate reactors

between 0 h and 24 h and between 72 h and 84 h where the 95% confidence interval of the bacterial rRNA relative abundance overlapped with the interval for the sum of the *Pseudomonas* and LGC-GPB rRNA relative abundance. These differences indicate that the selected probes did not account for a substantial portion of the bacterial community during the most acidic phase. The work reported here suggests that both the numbers and types of microorganisms present during the mesophilic-acidic phase differ dramatically from those present during the thermophilic-basic/neutral phase. It is clear that a more extensive characterization of the acidic phase community will be necessary to account for the unidentified rRNA genes.

The second piece of evidence suggesting that our knowledge of composting communities is limited is the low relative abundance of total bacterial rRNA genes in the early stages of composting. With the exceptions of 36 h and 72 h, the 95% confidence intervals for the mean relative abundance of bacterial populations did not overlap with the universal relative abundance. At the other time points, abundance of non-bacterial rRNA genes ranged from a low of 36% at 12 h to a high of 84% at 84 h. This resulted in a mean bacterial abundance that was only 52% of the total rRNA genes detected. This indicates that studying non-bacterial populations deserves a greater emphasis, especially during the earliest stages of the process. Previous PCR analysis using domain-specific primers on the same substrate showed the presence of yeast and other fungi, but not Archaea (Dees and Ghiorse 2001). It is also unlikely that the unidentified rRNA genes came from plant material, as preliminary results showed that the bead-beating technique used in this study to extract DNA from compost did not result in the co-extraction of plant DNA (Howeler et al. 2003; Malik et al. 1994). When coupled with the previous PCR analysis of the same DNA template (Dees and Ghiorse 2001), the limited amount of bacterial 16S rRNA genes, as a percentage of the total, suggests the possibility that fungi and possibly other microscopic eukaryotes may have accounted for much of the non-bacterial rRNA genes detected. It is therefore possible that fungi play a more significant role during the initial phase of composting than previously thought. The presence of fungi at the temperatures and pHs shown in Fig. 2 is plausible, considering a previous study that found that fungi were common early in the process and were later replaced by thermophilic bacteria after the matrix acidity had been reduced (Choi and Park 1998). Several studies confirm the presence of fungal populations such as the yeast *Candida* sp. (Peters et al. 2000) and the aeroallergen and opportunistic pathogen *Aspergillus fumigatus* (Strom 1985b) in compost. The limited attention given by researchers to the role that fungi play in compost may represent a systematic bias that has arisen from the use of bacteria-specific primers in PCR-based investigations of compost microbial communities (Blanc et al. 1999; Dees and Ghiorse 2001; Ishii et al. 2000; Malik et al. 1994; Pedro et al. 2001; Peters et al. 2000; Schloss et al. 2003a,b).

Previous culture-dependent and culture-independent composting studies have detected LAB and *Pseudomonas* sp.; however, those studies concluded that *Bacillus* sp. dominate composting microbial ecology (Dees and Ghiorse 2001; Ishii et al. 2000; Peters et al. 2000; Strom 1985b). The data presented here suggest, however, that even when *Bacillus* sp.-type genes are present, they only represented a maximum of one-half of the total LGC-GPB. The only LGC-GPB reported in the composting literature are LAB and *Bacillus* sp. (Blanc et al. 1999; Dees and Ghiorse 2001; Ishii et al. 2000; Pedro et al. 2001; Peters et al. 2000; Strom 1985a). If one assumes that the non-*Bacillus* sp. portion of the LGC-GPB-type genes are from LAB, the relative abundance of LAB-type genes exceeded the amount of *Bacillus*-type rRNA genes by between 1.5-fold and 3-fold. As many *Bacillus* sp. have been shown to possess upwards of ten copies of the genes encoding rRNA (Shaver et al. 2002), the true ratio of LAB to *Bacillus*-type bacteria may be even greater. Further analysis is necessary to determine if the non-*Bacillus* rRNA genes detected with the LGC-GPB probe are phylogenetically related to LAB or other, as yet unreported, compost bacteria. More work is also needed to determine if the ratio of *Bacillus* to other LGC-GPB is maintained beyond 96 h.

The hierarchical probing strategy used in this work resulted in the collection of data that appears to be internally consistent except at 96 h, when the LGC-GPB relative abundance accounted for 135% of the total rRNA genes. Considering the other relative abundance levels at 96 h were similar to those at 72 h and 84 h, and the quality of the standard curve for the universal and *Bacillus* sp. (R^2>0.99, Fig. 1), we assumed that the LGC-GPB relative abundance was spurious. Assuming that the relative abundance of the other probing signals are correct at

96 h and that we were unable to account for 25% of the bacterial 16S rRNA genes (based on the unaccountable portion from 72 h and 84 h), then we estimate that 40% of the universal signal at 96 h was from LGC-GPB-type genes. However, it is also possible that this time point may point to a methodological limitation.

Several factors limited the internal and external validity of our study. First, since composting is a highly variable process (Schloss and Walker 2001), we have historically used a SFW, dog food, as a substrate to minimize the variability introduced by substrate (Schloss and Walker 2000, 2001; Schloss et al. 2000, 2003a,b; VanderGheynst et al. 1997a, 1998; Walker et al. 1999). The nutritional composition of dog food is similar to food wastes, making it a useful substrate for a controlled study where our goal was to correlate basic changes in the microbial ecology with process-level changes using a generic substrate. Although the temperature profiles observed in the 500-ml laboratory scale reactors used in this study do not exactly mimic our larger scale compost reactors, they do follow the trends of rapid temperature increase over a period of 36–48 h. In addition, they allow for more effective control of experimental variability that cannot be achieved with large systems, and they allow us to systematically identify salient variables for future experiments. Second, a limitation of using DNA-based techniques is the finding that DNA may be long-lived in some environmental matrices (Romanowski et al. 1992) and therefore may not represent viable microorganisms. However, throughout this study, it was possible to observe discrete changes in *Bacillus*-type populations (Fig. 3). This would indicate that even contamination with recalcitrant spore DNA was apparently not an issue. A second limitation of using genomic DNA instead of rRNA is that there are typically only several copies of the rRNA genes in a genome although there may be hundreds of copies of each rRNA molecule in a cell. At a practical level, this limited yield of target nucleic acid per cell makes it difficult to use a large number of probes when using DNA as a target. As the SFW is a highly heterogeneous matrix, it would have been difficult to obtain rRNA molecules from the large number of samples used in this study. The choice to use DNA, however, reduced the number of probes we could use since the compost DNA was limiting. Although membrane stripping and reprobing was attempted, it differentially removed compost DNA from the membrane, making the results from further analyses questionable. Finally, the primary limitation of hybridization probing techniques is the quality of the probe. Probe selection for this study was guided by the results of previous studies, including one that included the same genomic DNA characterized here (Blanc et al. 1999; Dees and Ghiorse 2001; Ishii et al. 2000; Malik et al. 1994; Pedro et al. 2001; Peters et al. 2000; Schloss et al. 2003a,b; Strom 1985b). Although the bacteria-specific probe used in this study will not target phyla such as the *Planctomycetales* and *Verrucomicrobia*, representatives of these phyla have not been previously reported in compost. In spite of these various limitations, we were successful at finding correlations between physical process parameters and changes in population levels.

Few microbial ecology studies have been published from which both qualitative PCR-based results and quantitative hybridization results have been obtained using the same DNA templates (Buckley and Schmidt 2001; Chapelle et al. 2002; Salzman et al. 2002). None of these studies examined compost. Such studies permit a unique comparison of these two methods. PCR-based analysis was confirmed by the hybridization results in two of these studies (Buckley and Schmidt 2001; Chapelle et al. 2002) but it was not in the third (Salzman et al. 2002). Results of our previous qualitative fingerprinting analysis (Schloss et al. 2003a) suggested that a large population shift occurred during which LAB were replaced by *Bacillus* populations between 24 h and 72 h. In this present study, however, we found that the changes in relative abundance of *Bacillus* and LAB populations mirrored each other throughout much of the process, contradicting the PCR-based findings. Furthermore, although the previous analysis (Schloss et al. 2003a) suggested that *Pseudomonas*-type populations were present throughout the process, the presence/absence data from the PCR-based analysis did not give any hint of the approximately 12-fold change in relative abundance that was detected using probe hybridization. These discrepancies may be due in part to the well-reported biases found in PCR-based approaches (Hansen et al. 1998; Polz and Cavanaugh 1998; Suzuki and Giovannoni 1996) and differences in copy number between various bacteria (Shaver et al. 2002).

In our previous studies, we have worked towards developing mathematical models to integrate microorganisms into the heat-transfer and mass-transfer mechanisms and the microbial kinetics of the composting process (Higgins and Walker 2001; VanderGheynst et al. 1997c). With the exception of isolated conceptual models reported in the literature (Kaiser 1995), microbial biomass has not been linked to process kinetics. Although there is still much general work remaining, this study provides quantitative data that may be useful in the development of more complete composting models that are more biologically based. In addition, this study points us towards the acidic phase for further characterization of the bacterial community and the process as a whole for analysis of the non-bacterial community.

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