Spatial and temporal changes in the microbial community in an anaerobic swine waste treatment lagoon

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Abstract
Microorganisms are central to both the beneficial (organic degradation, nutrient removal, biogas production) and detrimental (odor production, pathogen contamination) effects of swine waste storage systems. In this study, both quantitative (real-time polymerase chain reaction) and qualitative (denaturing gradient gel electrophoresis, cloning, sequence analysis) molecular analyses were used to track spatial and temporal changes in the microbial community of swine slurry in the swine lagoon. The lagoon, located in a region of western Kentucky which has a humid, subtropical environment, was sampled on a monthly basis (n = 10) over a period of one year at four different depths (top, 51 cm from the top, 152 cm from the top, and bottom > 198 cm). The concentration and diversity of Bacteroides sp. was seasonal (up to 90% decrease between March and June). Hespellia sp. and other clostridial species, on the other hand, were endemic in the slurry (concentrations up to 1.0 × 10^9 cells mL^-1 slurry) regardless of time of the year or lagoon depth. Results suggest that there were seasonal effects on the microbial community in stored wastes may be (directly or indirectly) correlated with changes in malodor emissions from lagoons.

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1. Introduction

Over the last 30 years animal production has become increasingly intensive with fewer operations producing larger numbers of animals. As a consequence, the animal waste is produced and maintained in fewer facilities and in greater concentrations [1]. In swine production systems, this mixture of feces, urine, and wash water is stored in pits beneath the facility or in lagoons located adjacent to confinement areas. The manure is a valuable resource for crop fertilization and soil conditioning. However, there are also significant concerns associated with the handling of increasing volumes of swine wastes including malodor emissions and pathogen contamination. Land application of waste products may pose an additional risk to environmental resources through release of excessive nutrients, salts and organic materials, among others [2,3]. Additionally, long term or excessive application of manure may lead to eutrophication of water sources or soil deterioration.

With the national swine inventory exceeding 65 million [4] and steadily increasing, there is greater need to improve swine waste storage and handling to maximize the benefits while mitigating any negative consequences of animal waste utilization. Microorganisms are central to both the beneficial (organic degradation, nutrient removal, biogas production) and detrimental (odor production, pathogen contamination) effects of swine waste storage systems. Therefore, understanding more about how the microbial community functions in stored swine manure should aid in developing better means for management and usage of waste materials.

The microbial community in swine slurry has been characterized by traditional culture methods [5], 16S rDNA clone sequence analysis [6,7] or other molecular microbial community analysis techniques [8–10]. These studies showed that the slurry community is dominated by low G+C gram positive bacteria (predominantly Clostridium–Eubacterium species) and Bacteroides from the gram-negative bacteria. This is similar to the population in swine feces in which 81% of clones belonged to the low G+C gram positive bacteria and 11% to Bacteroides [11]. As in most
other environments, culturability is very low in swine slurry (20\% or less) [5]. Most of the culturable organisms can be identified (95–97\% 16S rDNA sequence similarity to known organisms) while less than 50\% of clones from 16S rRNA gene libraries are closely related to those of known organisms [6,7,10]. Others have found pathogenic organisms including *Salmonella* sp. and *Campylobacter* sp. in slurries [12,13]. These recent studies have provided insight into the dominant species in swine slurry and data on the occurrence of pathogens, but more information is needed to understand how the microbial community responds to chemical, physical and environmental fluctuations in these waste storage systems.

More than 150 chemical compounds can be emitted from waste storage systems including small-chain volatile fatty acids, phenols, indoles, ammonia and hydrogen sulfide [9,14,15]. Many of these compounds are problematic due to their low threshold for detection and difficulties involved in identifying any direct relationship between odor production from livestock facilities and concentrations in air emissions. For example, hydrogen sulfide and other sulfur-containing compounds are responsible for as much as half of the odorants in swine waste [15,16]. However, the form and concentration of the emissions depends on a complex interaction of factors including transport efficiency, lagoon physical parameters (temperature and pH) and differences in microbiological populations and activities [3,15].

Many of these malodorous compounds are generated as a consequence of metabolism of waste components by microorganisms [3,14,17]. However, much less is known about the microbial community dynamics in waste storage systems than is known about the physical or chemical parameters within those systems [9,18]. Merrill and Halverson [9] evaluated variations in microbial communities associated with stored swine manure in lagoons. They found that there were seasonal changes in community profiles that may be linked with malodor emissions. However, the method they used for community analysis (fatty acid methyl ester (FAME)) did not permit identification of specific organisms that were associated with shifts in the community. Peu et al. [10] used PCR-single-strand conformation polymorphism (SSCP) to show that the microbial community in anaerobic systems stabilized within 2–3 weeks of storage in anaerobic tanks or lagoons, but shifts significantly on movement from one location to another (i.e., from pit storage to lagoon storage). This kind of information regarding the response of microbial populations to physical and environmental shifts within stored swine slurries is essential for development of systems that will function effectively to reduce odors and pathogens. In this study, molecular analyses (denaturing gradient gel electrophoresis (DGGE), quantitative, real-time PCR (QRT-PCR), cloning and sequence analysis) were used to evaluate temporal (one year of sampling) and spatial (four depths) changes in microbial communities in a swine manure storage lagoon.

2. Materials and methods

2.1. Sample collection and chemical analyses of swine lagoon samples

Swine slurry samples were taken from an anaerobic swine lagoon (0.4 ha) from a farrowing operation (~2000 sows). The lagoon is located in a region of western Kentucky which has a humid, subtropical environment with an average temperature of 19 °C. The area receives an average rainfall of 131 cm per year. The slurry, a mixture of feces, urine and water used to clean stalls, was stored for about 2 weeks in pits located under the house before being evacuated by a flushing procedure to the lagoon. Slurry samples from the lagoon were taken at four different depths (top, 51 cm from the top, 152 cm from the top, and bottom >198 cm) with a pulley system designed so samples can be retrieved at specific depths. The bottom of the lagoon ranged from 250 cm to 300 cm, except at drawdown. Slurry from the lagoon was land-applied during September 2006 and April 2007 resulting in a drawdown of the lagoon depth to 124 cm and 170 cm, respectively. Samples were taken from all depths approximately every month (n = 10) from June 2006 to May 2007. Lagoon temperature was monitored with two HOBO water temperature probes (Onset Computer Corporation, Bourne, MA). One was floated on the surface of the lagoon while the other was weighted with lead sinkers and sunk to the bottom of the lagoon. Both probes were moored to an anchor at the edge of the lagoon for retrieval.

A combination electrode (Flue Scientist, Hampton, NH) was used to determine pH. Chemical oxygen demand (COD) was determined using dichromate reactor digestion kit for high strength wastewater (Chemetrix Inc., Calverton, VA). One ml samples were digested for 2 h at 150 °C and after the samples cooled, absorbance was read at 620 nm. Total dissolved solids (TDS) were determined according to standard method [19]. TDS were filterable-solids fraction which consists of both organic and inorganic molecules and ions that are present in true solution in water. Total organic carbon (TOC) was determined by combustion [20] of the lagoon water using a Vario Max CN analyzer (Elementar Americas, Inc. Mt. Laurel, NJ).

2.2. DNA extraction and PCR amplification

DNA was extracted from swine lagoon slurry samples (0.5 ml) in duplicate for each strain on each sampling day using the FastDNA® Spin kit for soils (MP Biomedical, Solon, OH, USA) according to manufacturer’s specifications. Specific PCR primer sets were used to target the 16S rRNA gene of the total microbial population, the *Clostridia/Eubacteria* (CE) group, or the *Bacteroides/Prevotella/Porphyrmonas* (BPP) group in the swine lagoon (Table 1). One to 10 ng of DNA extract was used as PCR template with the appropriate primer set (with 800 nM of each primer).

2.3. DGGE

Total bacterial community 16S rDNA was amplified from the bulk DNA extracts (using 1–10 ng of DNA per PCR) with the general bacterial PCR primer set 341F-GC and 907R, using a previously described PCR protocol [21] in a PTC-200 DNA thermal cycler (MJ Research, Las Vegas, NV). To evaluate the community structure of two of the dominant swine lagoon slurry bacterial groups the *Clostridia/Eubacteria* [22] or the *Bacteroides/Prevotella/Porphyromonas* [23], group-specific PCR primer sets were used according to published protocols (Table 1). The GC designation on the 341F, BPP-F-GC, and W109-R-GC primers represents a 40 bp GC rich region on the 5’ end of the primer necessary to prevent complete denaturation of the DNA strands during electrophoresis. Sequences were amplified using HotStarTaq® MasterMix Kit (Qiagen Inc, Valencia, CA), with 800 nM each primer. The above PCR protocols were modified, adding an additional heating phase (95 °C for 15 min) at the beginning of the reaction, as suggested by the manufacturer of the HotStarTaq® polymerase.

DGGE was used to separate and characterize 16S rRNA gene sequences by using a gradient of denaturants (100% denaturant solution consisting of a combination of 40% [vol/vol] formamide and 7 M urea) in a polyacrylamide gel (37.5:1) to separate DNA fragments according to melting behavior (i.e. sequence, melting domains). GelBond PAG Film (Cambrex BioSciences Rockland, MA) was used during pouring of the DGGE gels to allow for easier manipulation of the polyacrylamide gel after electrophoresis.
Twenty-five μL of PCR product was electrophoresed through a 30–60% (total bacteria and CE group) or 30–50% (BPP group) denaturing gradient according to Nübel et al. [24] for 4 h at 200 V in a Bio-Rad DCode universal mutation detection (Bio-Rad Laboratories, Hercules, CA). The DGGE gels were stained with the Bio-Rad Silver Stain kit according to the manufacturer’s specifications, and the images were captured using an Epson Perfection 4990 Photo Scanner (Epson, Long Beach, CA).

DGGE fingerprint analysis was performed using the Fingerprint II software program (Bio-Rad Laboratories, Hercules, CA) using the Basic, Clustering Analysis, Comparative Quantification and Polymorphism Analysis and Dimensioning Techniques modules. The gel images were imported into the software and analyzed according to the manufacturer’s specifications. The software package performed band matching between the fingerprints, allowing for the principle component analysis (PCA).

### 2.4. DNA sequencing and phylogenetic analysis

For DGGE band sequencing, each band was excised using a sterile scalpel and forceps and placed into 150 μL of 10 mM Tris buffer. 0.1 mm Zirconia/Silica beads (BioSpec Products, Inc., Bartlesville, OK) were added to each tube, and the samples were placed in a Fast Prep FP120 (Q-BIOgene, Irvine, CA) for 1 min at a speed of 6000 (14,000) rpm. The resultant PCR product was cloned into the pCR2.1-TOPO plasmid using a TA TOPO Cloning Kit (Invitrogen, Carlsbad, CA) according to manufacturer’s specifications and sent to USDA-ARS MSA Genomics Laboratory (Stoneville, MS) for sequencing. DGGE band sequences were submitted to the BLASTn 2.2 search engine [25] to obtain putative phylogenetic assignments for each band. The DGGE band sequences, combined with appropriate known sequences (depending on DGGE bands analyzed) from the GenBank database, were aligned using MEGA version 3.1 [26]. The alignment files were used to create bootstrapped (n = 1000) Neighbor-joining trees, using the Kimura 2-parameter model in the MEGA version 3.1 software package. The classifier function of the Ribosomal Database Project II (RDP) [27] was used to assign 16S rRNA gene sequences to the RDP taxonomical hierarchy at the phylum, order and family levels of resolution. A total of 48 sequences were submitted to the GenBank database, and were assigned the accession numbers of EU834071–EU834119.

### 2.5. QRT-PCR

QRT-PCR analysis was carried out as previously described for total cells (16S rRNA gene copies) [28] and for all Bacteroides spp. [29] using the Qiagen HotStarTaq MasterMix (Qiagen, Valencia, CA) and primers and probes shown in Table 1. The amplification mixture contained 3.0 mM MgCl2, 600 nM each primer, 200 nM of probe and sample DNA or standard (from 102 to 108 copies). For 16S rRNA gene copies, the QRT-PCR program was 15 min at 95 °C, 39 cycles at 95 °C for 1 s, 58 °C for 45 s, and 72 °C for 45 s. For Bacteroides sp., the QRT-PCR program was 15 min at 95 °C, 49 cycles at 95 °C for 1 s, 53 °C for 30 s, and 60 °C for 45 s. Cell concentrations were calculated by dividing the copy number per mL of slurry by 4.0 or 6.0, the average copy number of 16S rRNA genes in all cells or in Bacteroides cells, respectively [30].

QRT-PCR analysis of the CE group was carried out using the primers used for DGGE analysis, but without the GC clamp (Table 1) in a QuantiTect™ SYBR® Green (Qiagen, Valencia, CA) PCR reaction. The amplification mixture contained 800 nM each primer, sample DNA or standard (101–106 copies). The QRT-PCR program was 15 min at 95 °C, followed by 45 cycles of 95 °C for 30 s, 53 °C for 30 s, and 72 °C for 60 s. Since the SYBR Green dye binds to all double stranded DNA, a melting curve was performed after the reaction to ensure proper amplification had occurred. The melting curve parameters were temperatures ranging from 50 °C to 90 °C, with reads every 0.2 °C after a 1 s hold.

QRT-PCR assays were run on the DNA Engine Opticon 2 (MJ Research, Inc., Waltham, MA) in a total volume of 25 μL. Baseline values were set as the lowest fluorescence signal measured in the well over all cycles. The baseline was subtracted from all values and the threshold was set to one standard deviation of the mean. All PCR runs included duplicates of standards and control reactions without template. Standard DNA consisted of plasmid PCR 2.1 vector (Invitrogen, Carlsbad, CA) carrying the appropriate insert for

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**Table 1**

<table>
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<tr>
<th>Target</th>
<th>Oligo Name</th>
<th>Sequence (5’-3’)a,b</th>
<th>Tm (°C)</th>
<th>Insert size (Abp)</th>
<th>Application</th>
<th>Reference</th>
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<tr>
<td>All Bacteria</td>
<td>341-F</td>
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<td>64.7</td>
<td>566</td>
<td>DGGE</td>
<td>[21]</td>
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<td>341-F-GC</td>
<td>cgc ccg ccq ccq ccc ggc gtc cgc ccc ccc ccq cgg CTA CGG GAG</td>
<td>87.8</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>907-R</td>
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<tr>
<td>All Bacteria</td>
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<td>ATG CTC GTC GTC AGC</td>
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<td>337</td>
<td>QRT-PCR</td>
<td>[28]</td>
</tr>
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<td></td>
<td>B16s-Taq115-F</td>
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<td>Bacteroides–Prevotella–Porphyromonas</td>
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<td>418</td>
<td>DGGE</td>
<td>[23]</td>
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<td></td>
<td>BPP-GC</td>
<td>cgc ccq ccq ccc ggc ccc gtc cgq ccc ccc ccc gca AGC TCC CCC</td>
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<td></td>
<td></td>
<td></td>
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<tr>
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<td>BPP-R</td>
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<td>552</td>
<td>DGGE &amp; QRT-PCR</td>
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<td></td>
<td>W109-R</td>
<td>CCC TTT ACA CCC AGT AA</td>
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<td></td>
<td>W109-R-GC</td>
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<td>QRT-PCR</td>
<td>[29]</td>
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<tr>
<td></td>
<td>Allbac412-R</td>
<td>CCCTACTGTGGCTTGTCAC</td>
<td>63.5</td>
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<tr>
<td></td>
<td>Allbac373-Bhq-R</td>
<td>(FAM)GCTATTGAGAATATCTGCGGTCGC (BHQ-1)</td>
<td>74.7</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

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a Degeneracy codes: R = A or G, Y = C or T, K = G or T, M = A or C, S = G or C, W = A or T, H = A or C, D = G or A, V = A or C or G.

b Lowercase sequence represents the GC clamped portion of the primer.

Includes Clusters I, III, IV, XIIa, XIVb and rumen Clostridia spp.
the given assay. DNA concentrations in each extraction were determined using the Hoechst 33258 nucleic acid stain (Invitrogen, Carlsbad, CA) and measured with a Hoefer DyNA Quant 200 fluorometer (Amersham Biosciences, San Francisco, CA) according to manufacturer’s instructions.

3. Results and discussion

3.1. Spatial and temporal changes in total cell numbers

The distribution of cells over the depth of the lagoon was fairly homogenous (Fig. 1A). Total cell numbers averaged $1.4 \pm 0.7 \times 10^{8}$, $1.1 \pm 0.6 \times 10^{8}$, $2.7 \pm 1.3 \times 10^{8}$, and $1.7 \pm 1.3 \times 10^{9}$ cells mL$^{-1}$ slurry at the top, 51 cm, 152 cm and at the bottom (>198 cm) of the lagoon. The similarity in cell concentrations at the top and bottom of the lagoon occurred despite the fact that all chemical parameters (except pH) were higher at the bottom of the lagoon than at the other depths (Table 2). Although, total cell numbers from this study were lower than those obtained by microscopic cell counts of slurry (0.6–1.0 $\times 10^{9}$ cells mL$^{-1}$ slurry), they were the same or higher than culturable counts ($1 \times 10^{6}$–$2 \times 10^{8}$ cells mL$^{-1}$ slurry) [5,8,31].

Total cell numbers in samples taken from 51 cm and from the lagoon bottom increased significantly ($p < 0.05$; over 87% and 79%, respectively) between Mar and Aug (Fig. 1A). Temperature is the most likely cause of the increase in total cell numbers in the slurry rather than a change in livestock management since this is a farrowing operation and as such is highly stable in its routine. Slurry temperatures during summer months (June, July and Aug) averaged 29.3 ± 1.2 °C, while winter month temperatures (Dec thru Feb) averaged 8.1 ± 2.3 °C (Fig. 2). Temperatures in spring (Mar, April and May) and fall (Sept, Oct, and Nov) were transitional and were therefore more variable over the seasonal span (18.0 ± 4.7 °C and 18.2 ± 5.2 °C, respectively). The change in cell numbers between Mar and Aug tracked with an increase in slurry temperature of 16–18 °C (Fig. 2), but no consistent changes in chemical or physical lagoon characteristics over that time period (data not shown). UPGMA analysis of DGGE banding patterns showed that samples taken on the same date exhibited more similarity among themselves (greater than 70%) than with samples taken on other dates, regardless of depth (data not shown). These results and those from the QRT-PCR suggest that there is greater temporal variability in the general microbial population than spatial variability. Samples taken from the top of the lagoon and from 152 cm did not change significantly ($p > 0.05$). It is uncertain why the mid-levels of the lagoon would be less impacted by the seasonal change than would the bottom.

There was another significant ($p < 0.01$) increase in cell numbers in Sept. This increase may be partially associated with the drawdown of the lagoon which occurred during that month. During this time there was also an increase in COD (25%), TDS (41%) and TOC (29%). However, by Sept there had already been a significant increase in cell numbers over the previous 3–4 months (Fig. 1A). Furthermore, there was also a lagoon drawdown in April and there was no corresponding increase in cell numbers in samples taken following the event in May (Fig. 1A). The change in chemical parameters, however, was also more modest (5–10%) following the drawdown in April. Based on the data obtained in this study, it is difficult to determine if there was an association between the increases in cell numbers observed in Sept and changes in the physical or chemical structure that resulted from the drawdown of the lagoon. However, the data do suggest that there was

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Table 2

<table>
<thead>
<tr>
<th>Depth (cm)</th>
<th>COD (mg L$^{-1}$)</th>
<th>TDS (mg L$^{-1}$)</th>
<th>TOC (mg L$^{-1}$)</th>
<th>pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Top</td>
<td>3809 ± 737</td>
<td>3796 ± 878</td>
<td>1968 ± 768</td>
<td>7.59 ± 0.39</td>
</tr>
<tr>
<td>51</td>
<td>3516 ± 440</td>
<td>4277 ± 998</td>
<td>1576 ± 538</td>
<td>7.62 ± 0.36</td>
</tr>
<tr>
<td>152</td>
<td>3946 ± 365</td>
<td>4450 ± 1006</td>
<td>4474 ± 5562</td>
<td>7.76 ± 0.36</td>
</tr>
<tr>
<td>Bottom</td>
<td>50796 ± 13742</td>
<td>11119 ± 5156</td>
<td>15905 ± 8397</td>
<td>7.52 ± 0.27</td>
</tr>
</tbody>
</table>

Values represent yearly average ± standard deviation.
COD, Chemical oxygen demand; TDS, Total dissolved solids; and TOC, Total organic carbon.

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![Fig. 1](image1.png)

**Fig. 1.** Log$_{10}$ of the concentration of bacterial cell numbers as determined by quantitative, real-time PCR (QRT-PCR) targeting (A) total cells (16S rRNA gene), (B) Clostridia–Eubacteria (CE), and (C) Bacteroides–Porphyromonas–Prevotella (BPP) groups. Values are in cells per mL of slurry at the top (●), 51 cm (▲), 152 cm (■) or bottom (▲) of the lagoon. It was assumed that the 16S rRNA gene was present in 4 copies, the CE targeted region was present in 11 copies, and the BPP targeted region was present in 6 copies [30]. Error bars represent the standard deviation of duplicate samples each also run in duplicate (four replicates).

![Fig. 2](image2.png)

**Fig. 2.** Average monthly air ( ), surface ( ) and sludge ( ) temperature (°C).
a significant increase in microbial cell numbers over the course of the spring and summer seasons.

DGGE analysis was utilized to evaluate changes in the community profile of the bacterial population by depth and over time. PCA of the DGGE banding pattern served to reduce the dimensionality of the data and permit graphic visualization of spatial and temporal changes in the community profiles (Fig. 3A). PCA of the DGGE pattern for the 16S community analysis showed that the community profiles were divided (Factor 1, explaining 22% of the difference among samples) between samples taken in spring and summer and those taken during cold months (Oct through Mar) (Fig. 3A). The second factor (16% the difference among samples) separated Sept (all samples), Oct (top) and Feb (152 cm and bottom) samples. Other than these transition periods, there were no differences based on sampling depth (Fig. 3B). There was a corresponding difference in DGGE band number observed for the warm months (mean bands = 12) versus the cold months (mean bands = 19). The impact of temperature transitions has also been found in other studies, for example, Yu and Mohn [32] found that changes in temperature in an aerated pulp and paper lagoon correlated with the most abrupt changes in the community structure. Merrill and Halverson (2002) found that the seasonal change in FAME profiles of swine slurry in concrete storage systems occurred in summer during the period of peak system temperature. Similarly, the transitional DGGE patterns for the fall (separated on PC2) corresponded to the months that showed the largest shifts in temperature during the course of the year (Fig. 2). Therefore, temperature appeared to have a significant impact on community dynamics based on both QRT-PCR analysis of total cell numbers and on the DGGE community profiling.

3.2. Phylogenetic analysis of DGGE band sequences

Twenty-nine of the dominant DGGE bands were cloned and sequenced (Fig. 4). Nineteen (65%) of the cloned 16S rRNA gene sequences were similar (90% or greater) to database sequences from Firmicutes; thirteen (45%) of which grouped with the Clostridiales. Not surprisingly, the clostridial clone sequences matched most closely to other uncultured clones from swine pits, biogas reactors, sludge treatment or other anaerobic waste treatment systems [33–36] (Fig. 4). Six clones grouped with the Bacillus–Lactobacillus–Streptococcus (21%) sub-group and matched other clones from studies of the human gut or swine manure compost [33,37]. These Bacillales-related sequences were 90% similar to Lysinibacillus sp. or Ureabacillus sp. RDP classified these sequences as Erysipelothrix sp., which causes erysipelas an acute or chronic septicemia in swine [38]. However, the DGGE band sequences were less than 80% similar to those of Erysipelothrix sp. sequences from GenBank. Eight other clone sequences (28%) aligned with those from the gram-negative BPP group. These sequences matched uncultured clones from swine lagoons, anaerobic digester or sediment microcosms [39]. One cloned band sequence grouped with the Spirochaetales and one with Burkholderiales (Fig. 4). The grouping and distribution of clones predominantly between the Clostridiales, Bacillales, and the BPP groups was similar to that found in the swine GI tract [11] and in swine slurries [5–8]. Some species common to the swine gastrointestinal tract (i.e., Lactobacillus) were absent from the 16S DGGE gel band sequences [11]. However, the microbial community in swine slurry would not be expected to mimic that of the swine intestine given the fact that both physical conditions (temperature, frequency of emptying, retention time) and chemical makeup (urine, feces, barn debris, wash water) are quite different in the lagoon [5–8].

The seven most common and distinct DGGE bands (16S Bands 18, 8, 6, 26, 15, 9 and 17) contained sequences that were similar to Clostridium butyricum, Hespellia stercorisuis, Aminobacterium sp., Acidaminococcus sp., and Peptinophilus sp. Clostridia are commonly found in swine slurry and are involved in fermentation of lipids, sugars and amino acids with concomitant production of malodorous volatile fatty acids (VFA) [8,40]. Hespellia sp. are a new group of non-spore-forming, strictly anaerobic organisms that form a cluster within the Clostridium cocoides group [36]. Those organisms were originally isolated from swine slurry pits. The band (B6, Fig. 4) corresponding to a sequence similar to H. stercorisuis (98% similarity) was present in all slurry samples that were taken. Three other clones containing common band sequences were similar to those from genera containing amino acid degraders (Aminobacterium sp., Acidaminococcus sp., and Peptinophilus sp.). Proteins are one of the main organic substrates in anaerobic slurries, therefore, it is not surprising that three of the dominant species are related to organisms that degrade amino acids in the rumen and in anaerobic wastewaters [41,42]. Amino acid degraders are important in anaerobic slurry as they are responsible for production of some of the most offensive compounds associated with swine odor [5]. Data from this study suggest that these species are dominant and common in the swine slurry regardless of lagoon depth or season.
Fig. 4. Neighbor-joining tree showing the phylogenetic relationship between DGGE band sequences (bold text) and 16S rRNA gene sequences retrieved from the GenBank database (accession numbers in parentheses). The tree represents the alignment of an approximately 600-bp region of sequences from DGGE bands and from GenBank. The scale bar represents 5% estimated change. The major phylogenetic groups found in this study are indicated along the right side of the figure. An archaea was used as an outgroup [Halobacterium salinarum (DQ465019)]. Numbers at each node indicate percentage of occurrences in 500 bootstrap iterations for values over 75%.
Based on the 16S rRNA gene sequence analysis of data from the cloning of DGGE gel bands, *Clostridia* sp. and *Bacteroides* sp. were the two largest groups in the slurry. Furthermore, correlation of the DGGE profile with the band sequences suggests that the difference in DGGE banding patterns between the warm months and the cool months in the 16S DGGE profile correlated with the absence of band sequences from several *Bacteroidales* and one band that matched *Sedimentibacter* sp. Others have found that using primers for specific microbial groups permits visualization of subdominant populations that may not be visible in the total bacterial profile [10, 23]. Therefore, we focused more specifically on the CE and BPP groups by using group-specific primers (Table 1) to obtain PCR products from the swine slurry extract to be used for DGGE analysis.

3.3. Spatial and temporal dynamics of Clostridiales populations

Amplification of swine lagoon DNA extract with primers targeting the CE group resulted in DGGE profiles in which the bands were concentrated at the top or bottom of the gel. Peu et al. [10] also found, using SSCP which separates DNA fragments based on size and secondary structure, that the CE sub-group represented a distinct cluster within the total SSCP profile. In this study, PCA of the DGGE banding profile for the CE group showed that the profile for the CE group was not as significantly influenced by time of year as was the general 16S rDNA DGGE pattern (Fig. 3B). The first axis (explaining 30% of variability in the data) separated all bottom samples (Fig. 3B) along with samples from Feb, Mar and May. This was due to fewer bands in the bottom samples (mean bands = 3) than in samples from the other depths (mean bands = 11). QRT-PCR analysis targeting the *C. coccoides/Hespellia* sp. group of CE sequences showed that cell concentrations corroborated the results of DGGE analysis. The concentration of the CE group was lower in the bottom samples, especially in Dec, Feb, Mar and May (Fig. 2B). This decrease did not correlate to changes in physical or chemical parameters during those times (i.e., COD, TOC, TDS or pH).

Ten bands from the CE group DGGE gel were excised, cloned and sequenced. Five of the ten cloned band sequences fell into groups that were similar to the *Clostridiales* sequences obtained from the 16S DGGE band sequences (Table 3). Four CE group band sequences were over 97% similar to *C. coccoides* and *Hespellia* sp. and two of the 16S rDNA band sequences (B6, B26) aligned with the same group (Fig. 4). Two CE group band sequences and two 16S rDNA band sequences matched over 98% to those from *Sedimentibacter* sp. and two other band sequences were similar to a *Clostridium disporicum* (DQ855943) strain that has been found in the swine hindgut, pork manure biosolids and in swine slurry [8, 43]. Two sequences matched an uncultured clone from swine lagoon slurry (AY953242), but were less than 90% similar to any known sequences in the GenBank database. A clone that was 99% similar to *Hespellia porcina* strain PPC80 (AF445239) was present in all of the samples and was one of the few bands present in the bottom samples. Two other clones matched those from swine slurry, and although not close to any cultured species they were most closely linked with the *Hespellia* group. Those two clones coupled with the four other clones from this library and two from the 16S library, as well as the QRT-PCR data which suggest that this group is ubiquitous within the slurry suggests that *Hespellia* species are important in swine slurry and the group should be studied further.

3.4. Spatial and temporal dynamics of Bacteroides sp. populations

The PCA of the DGGE profile produced by products from PCR amplification of swine slurry with BPP group-specific primers was much more distinctive than that of the CE group (Fig. 3C). Fall samples (with Aug) separated from the spring and winter months (Dec thru May), accounting for 41% of the variability in the data. On the second axis (17% of variability), June (and Aug Top) samples separated from the other warm and cold months (Fig. 3C). Ten bands from the BPP group DGGE profile were excised, cloned and sequenced. In contrast to the clone sequences from the analysis of the CE group, the BPP group sequences were distinct from the 16S rDNA sequences that aligned with the BPP group (Table 3). The eight cloned 16S DGGE band sequences matched most closely with the Porphyromonadaceae and Rikenellaceae families or the Sphingobacteria, while the clone sequences obtained from the BPP group DGGE gel aligned more closely with those from the Bacteroidaceae or Prevotellaceae families (Table 3). Two cloned band sequences that were present in all samples matched those associated with Bacteroidetes clones from fecal pollution [44] or from a study of swine gut microbiota [11]. Two cloned band sequences that were missing in warm month samples matched those from studies of the human and swine gut [11, 45]. Evaluation of the DGGE band profile suggests that the difference in DGGE banding patterns correlated with a lower diversity of

Table 3 Phylogenetic grouping of cloned DGGE bands.

<table>
<thead>
<tr>
<th>Group*</th>
<th>Universal</th>
<th>BPP</th>
<th>CE</th>
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<tbody>
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</tr>
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<tr>
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<td>Spirochaetales</td>
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</tbody>
</table>

BPP, Bacteroides, Prevotella, Porphyromonas; and CE, Clostridium—Eubacterium.

* Group based on Ribosome Database Project designation.
species in warm months (mean bands = 7) versus cold months (mean bands = 10). There was no apparent shift in the species present (i.e., no shift in the dominant bands) in the winter months, rather the differences in the DGGE profiles were due to the decrease in bands in the warm months. The significant shift in Bacteroides sp. in slurry was confirmed by QRT-PCR analysis. Bacteroides sp. were approximately 10% of the total population (averaging 1.0 × 10^7 cells mL^{-1} slurry) (Fig. 1C). In June, however, the Bacteroides sp. population dropped to 1% or less of the population at all depths relative to total 16S QRT-PCR analysis. By Aug, the concentration of Bacteroides sp. had increased by an order of magnitude at the top, 51 cm and 152 cm depths. This increase was delayed at the bottom of the lagoon; however, there was a two order of magnitude increase between June and Sept (Fig. 1C). This data correlates well with PCA of DGGE profiles which suggest that samples from Aug, Sept and Oct were distinct from Dec through May samples and that June separated from all of the other months. There was no effect of depth. Okabe and Shimazu [46], studying the effect of temperature and salinity on the die-off of strains of Bacteroides sp. had increased by an order of magnitude at the top, Bacteroides sp. to total 16S QRT-PCR analysis. By Aug, the concentration of Bacteroides sp. was found to be more diverse in winter months and present at high concentrations (Fig. 1C). Transitions in the Bacteroides populations seen in June, the concomitant increase in pH and the high temperatures during summer months may be associated with changes in the microbial community that correlate with reduced odor production from the lagoon in summer. These results suggest that shifts in the Bacteroides population correlate with the seasonal changes observed for odor production in the swine lagoon. These populations may be partially responsible for this effect in the lagoon either directly or working in synergy with other organisms. Although the link or significance cannot be determined from data obtained in this study, it does warrant further investigation. Based on this data, it seems that a Bacteroides population has a much more seasonal aspect to their presence in the lagoon than do the Clostridiales.

4. Conclusions

Data from this study show that there is a distinct seasonal effect in the microbial community in swine lagoon storage systems. Hespeluria sp. and other clostralid species appear to be endemic in the slurry, present at all depths and throughout the year. Based on sequence analysis of 16S DGGE bands, PCA analysis of BPP group DGGE profile and on the QRT-PCR analysis, the Bacteroides sp. appear to be seasonal in their distribution in the slurry. Therefore, Bacteroides may account for the seasonal effect in the 16S DGGE profile and perhaps play an important role (either directly or in synergy with other microbial groups) in malodor emissions from waste storage systems [50]. Future work should evaluate the role of this group in community dynamics and odor production in the lagoon system. Furthermore, trends in these large groups may give little about the changes or physiological ecology of important functional groups within the system (methanogens, acetogens, sulfate reducers). These groups may have a major impact on biomass conversion and odor production within the lagoon.

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References


