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Dynamics of a Pig Slurry Microbial Community during Anaerobic Storage and Management

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The microbial community of a pig slurry on a farm was monitored for 6 months using both molecular and cultural approaches. Sampling was carried out at all the different stages of effluent handling, from the rearing build-up to slurry spreading. Total DNA of each sample was extracted and analyzed by PCR–single-strand conformation polymorphism (SSCP) analysis using primers targeting the 16S rRNA genes from the archaeal and bacterial domains and also the *Eubacterium-Clostridium*, *Bacillus-Streptococcus-Lactobacillus*, and *Bacteroides-Prevotella* groups. A comparison of the SSCP profiles showed that there were rapid changes in the dominant bacterial community during the first 2 weeks of anaerobic storage and that the community was relatively stable thereafter. Several bacterial populations, identified as populations closely related to uncultured *Clostridium* and *Porphyromonas* and to *Lactobacillus* and *Streptococcus* cultured species commonly isolated from pig feces, remained present and dominant from the rearing build-up to the time of spreading. Enumeration of fecal indicators (enterococci and *Escherichia coli*) performed in parallel using cultural methods revealed the same trends. On the other hand, the archaeal community adapted slowly during pig slurry storage, and its diversity increased. A shift between two hydrogenotrophic methanogenic *Methanobrevibacter* populations from the storage pit to the pond was observed. Microorganisms present in pig slurry at the time of spreading could not be detected in soil after spreading by either molecular or cultural techniques, probably because of the detection limit inherent in the two techniques.

The majority of pigs produced in industrialized countries are raised in buildings that are especially designed for this type of husbandry. The livestock buildings are entirely covered and insulated, and the pigs are housed in stalls with concrete floors for easy evacuation of feces and urine. Pits located below the animals receive the excrement for temporary indoor storage. After a few weeks, the effluent is generally evacuated to a large outdoor storage tank, where it stays for 4 to 6 months until it can be spread onto arable land (5). Although this type of husbandry is a success for pig production, the different stages of waste management generate severe air, soil, and water pollution (5, 14, 24, 25, 46, 47). Moreover, spreading large amounts of manure onto fields raises questions about the sanitary aspects of such practices (5, 7, 26, 27, 30).

The pig slurry microbial community, which was previously characterized by cultural methods (15, 16, 38), has recently been characterized by molecular techniques (37, 43). Of the 10^9 to 10^{10} cells per ml present in pig slurry, only 10 to 20% could be cultured on various media based on pig slurry (8, 33). Moreover, less than 50% of the phylotypes observed in pig slurry by 16S rRNA gene analysis were closely related to known microorganisms (37, 43). Nevertheless, based on both cultural and molecular approaches, the pig slurry microbial community appears to be an anaerobic digestion ecosystem that is dominated by groups of fermentative bacteria, such as the *Eubacterium-Clostridium* (EC) and *Lactobacillus-Streptococcus* groups of low-G+C-content gram-positive bacteria and the *Bacteroides* group of gram-negative bacteria. The members of the domain *Archaea* are dominated by hydrogenotrophic methanogens, as well as by a group of uncultured *Archaea* whose function is unknown.

Despite the increasing interest now being paid to characterization of pig slurry microbiology (48), few workers have tried to evaluate the impact of pig slurry storage conditions on the composition of the slurry microbial community (2, 26, 27, 33, 49). In most of the studies the researchers used cultural methods and focused on the fate of pathogenic or fecal indicators (26, 27). These studies have shown that the numbers of fecal indicators and pathogenic bacteria decrease rapidly and exponentially with time during pig slurry storage. In contrast, analyses of the whole microbial community by cultural (2, 33) and molecular techniques (20) have suggested that a more static view is possible and that the dominant microbial groups are relatively stable. A more systematic analysis of the pig slurry microbial community dynamics in pig-rearing structures should contribute to our understanding of metabolic transformations which occur during slurry storage. The aim of this work was to monitor the dynamics of a pig slurry microbial community throughout an intensive production installation in order to evaluate the impact of each waste management unit on the pig

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TABLE 1. Sequences and target positions of the primers used in this study

Primer	Direction[a]	Primer sequence (5′–3′)	*E. coli* position[c]	Targeted 16S rRNA	Reference
W18	F	GAGTTTGATCMTGGCTCAG	9	*Bacteria*	13
W17	F	ATTCYGGTTGATCCYGSCRG	6	*Archaea*	13
W02	R	GNTACCTTGTTACGACTT	1509	Universal	42
W49	F	AGGTCCAGACTCCTACGGG	330	*Bacteria*	9
W96	F	TCCAGGCCCACGGGG	333	*Archaea*	9
W104	R	TTACCGCGGCTGCTGGCAC[b]	500	Universal	9
W31	R	TTACCGCGGCTGCTGGCAC	500	Universal	9
W108	R	ATTYCACCGCTACACATG	679	*Bacillus*, *Lactobacillus*, *Lactococcus*, *Pediococcus*, *Leuconostoc*, *Weissella*, *Streptococcus*, *Enterococcus*, *Staphylococcus*	41
W109	R	CCCTTTACACCCAGTAA	561	*Eubacterium*, *Clostridiaceae* (clusters I, III, IV, XIVa, XIVb, Rumen)	40
W112	R	TCACCGTTGCCGGCGTACTC	887	*Prevotella*, *Bacteroides*, *Porphyromonas*	45

[a] F, forward; R, reverse.
[b] Labeled with the fluorophore 6-carboxyfluorescein.
[c] Data from reference 4.

slurry microbial community. Dominant bacterial and archaeal communities were monitored by 16S rRNA gene-targeted PCR amplification and single-strand conformation polymorphism (SSCP) analysis (18, 28, 36, 50). Fecal indicators (enterococci and *Escherichia coli*) were monitored by using cultural methods. The most prominent microbial populations observed by PCR-SSCP analysis were identified by DNA sequence analysis.

MATERIALS AND METHODS

Farm description and sampling. Sampling was carried out between November 2002 and June 2003 on an intensive pig-fattening farm located in southwest France near Albi. This farm breeds 220 Broad-White sows plus finishers that weigh up to 100 kg (live weight) and produces about 4,500 m^3 of pig slurry per year. On this farm, pig slurry is stored for about 2 weeks in pits located under stalls before it is evacuated by a flushing procedure to a large, outside, covered, homogenized tank with a total capacity of 800 m^3. The flushing procedure is operated by pumping the pig slurry from the outside storage tank back into the rearing house pits in order to avoid accumulation of solids in the indoor pits. After a few weeks of storage in the outdoor tank, the pig slurry is treated in a second stage with an auger press to separate the liquid and solid parts. The liquid part is evacuated to a pond with a capacity of 2,500 m^3, while the solid part is stored in an open building. After storage for 4 to 6 months, the liquid from the pond is spread on farmland at a level of about 170 kg $hectare^{-1}$ (about 69 kg $acre^{-1}$) of organic nitrogen from livestock waste.

A total of 28 samples were taken at the different stages of the pig slurry accumulation and handling process. Slurry samples were collected at different locations within the indoor pits with a sampling cane and then mixed together. Samples from the outdoor storage tank were taken every 2 or 3 weeks after 1 h of homogenization, starting with the initial filling in December 2002. The pond was sampled four times during refilling in March 2003. Soil sampling was carried out 15 days before and immediately before pig slurry spreading. Samples were also taken just after spreading and during a subsequent 2-month period. At each sampling time, nine core samples from a depth of 0 to 15 cm were obtained with a drill and thoroughly mixed. After mixing, a subsample consisting of about 1 kg of soil was collected. All samples were brought to the laboratory within 12 h.

Physicochemical characterization of pig slurry samples. pH was determined by direct measurement with a glass electrode pH meter. Total ammonia nitrogen and total Kjeldahl nitrogen were analyzed by steam distillation coupled with a titration unit. For total nitrogen, prior mineralization at 350°C was required to reduce all nitrogen forms to ammonium. The dry matter was analyzed by drying samples at 105°C until the weight was constant. The volatile fatty acid content was analyzed by high-performance liquid chromatography (29).

Enumeration of classical fecal indicators by cultural techniques. Enumeration was performed by using 25 g of solid samples or, for liquid samples, a 25-ml pellet obtained by centrifugation at 8,000 rpm for 12 min of 500 ml of pig slurry. Samples were homogenized in a stomacher bag with 225 ml of tryptone-salt solution for 10 min. Plate counts of enterococci were determined by using kanamycin esculin azide agar base (Oxoid) that was incubated at 37°C for 24 h. *E. coli* counts were determined using Select *E. coli* Count Plate Petrifilm (3 M) that was incubated at 44°C for 24 h. The data were expressed as numbers of CFU per gram (dry weight). Means of unadjusted variables were compared using Student's *t* test.

DNA extraction. Liquid pig slurry samples (10 ml) were centrifuged for 10 min at 17,500 × *g*. Each pellet was homogenized in a mortar containing 4 ml of 4 M guanidine thiocyanate–0.1 M Tris-HCl (pH 7.5) and 0.6 ml of 10% *N*-lauroyl sarcosine. Fresh soil samples were sieved using a 0.04-cm^2 mesh. Ten-gram aliquots were crushed in a mortar in the same way with 8 ml of guanidine thiocyanate and 1.2 ml of *N*-lauroyl sarcosine. Four 0.5-ml aliquots were transferred into microtubes and immediately stored at −20°C. Nucleic acids were extracted as described previously (13) except for the soil samples, to which 0.2 ml of aluminum ammonium bisulfate [$AlNH_4(SO_4)_2$] was added at the beginning of the extraction procedure in order to eliminate most PCR inhibitors (3).

Direct and nested PCR-SSCP analysis of microbial 16S rRNA genes. The pig slurry bacterial and archaeal communities were analyzed by PCR amplification of the 16S rRNA gene V3 region using the W49-W104 and W96-W104 primer pairs, respectively (Table 1), and *Pfu* Turbo DNA polymerase (Stratagene) as described by Chachkhiani et al. (6). Twenty-five cycles of amplification were performed with a GeneAmp 9700 thermocycler (PE Applied Biosystems). The resulting PCR products were then separated by SSCP capillary electrophoresis with an ABI 310 genetic analyzer (Applied Biosystems) as described by Delbes et al. (10).

A more precise analysis of dominant phylogenetic groups was carried out by nested PCR. In the first step, the 16S rRNA genes of the targeted bacterial group were amplified using AccuPrime *Taq* DNA polymerase (Invitrogen, The Netherlands) and a group-specific primer (W108, W109, or W112) along with a bacterial domain primer (W18). On the basis of the June 2002 ARB database release (updated with deposited sequences related to pig fecal flora and pig slurry flora [19, 31, 37, 43]), primer W108 targets 60% of the sequences belonging to the *Bacillus-Streptococcus-Lactobacillus* (BSL) group (41), primer W109 targets 80% of the sequences belonging to the *Eubacterium-Clostridium* (EC) group (40), and W112 targets 85% of the sequences belonging to the *Bacteroides-Prevotella* (BP) group (45). PCRs were performed according to recommendations of the supplier (Invitrogen). The reaction mixtures contained about 100 ng of sample genomic DNA, 1× AccuPrime *Taq* polymerase buffer, 50 ng of each primer, 2.5 U of AccuPrime *Taq* polymerase (Invitrogen), and enough H_2O to bring the volume to 25 μl. The PCR conditions were as follows: denaturation for 2 min at 94°C and then 30 cycles of 30 s at 94°C, 30 s at the annealing temperature, and 90 s at 68°C. No final elongation was performed, as recommended by the supplier (Invitrogen). The reaction was stopped by cooling the mixture to 4°C. The annealing temperatures of the primers were 52°C for W108, 50°C for W109, and 61°C for W112. Amplification product sizes were confirmed by electrophoresis on a 0.7% (wt/vol) agarose gel. Products obtained from the PCR were diluted 100-fold, and 1 μl of each product was used as a template for a

PCR-SSCP analysis carried out using the primers and conditions described above.

Clone library, sequencing, and identification of dominant 16S rRNA gene fragments. The dominant peaks in the SSCP profiles for pig slurry samples collected from the storage tank on day 21 were identified. For the *Archaea*, the 16S rRNA gene V3 region was amplified from total DNA using the red*Taq* DNA polymerase (Sigma, France) and the archaeal W96-W31 primer pair (Table 1). For each targeted microbial group (BSL, BP, and EC groups), the AccuPrime *Taq* polymerase PCR products obtained as described above were amplified again using bacterial primers W49 and W31 (Table 1). These amplifications generated the same DNA fragments that the amplifications performed for the SSCP analysis generated, except that, since the W31 primer was not labeled, the DNA fragments could be cloned into *E. coli* using a TOPO-TA vector cloning kit (Invitrogen) to generate 16S rRNA gene V3 libraries. Then about 40 clones from each library (45 clones for the BSL library, 42 clones for the BP library, 52 clones for the EC library, and 40 clones for the *Archaea* library) were randomly picked, and their inserts were screened by PCR-SSCP analysis as described previously (6). Inserts that comigrated with distinguishable peaks from the total DNA SSCP profile were sequenced to finalize the identification of peaks. DNA sequences were obtained using a dye terminator cycle sequencing Ready Reaction kit (Big Dye Terminator; Applied Biosystems) and a 373A Genetic Analyzer from Applied Biosystems. DNA sequences were identified by comparison with their closest relatives available in databases using BLAST from the National Center for Biotechnology Information and the Ribosomal Database Project (1, 23) and by fitting the sequences into preexisting trees using the parsimony interactive tool from the ARB software package (22).

Nucleotide sequence accession numbers. Sequences have been deposited in the EMBL database under accession numbers AM229418 to AM229448.

RESULTS

Physical and chemical characteristics of the effluent. Pig slurry collected from the storage tank and pond had chemical characteristics similar to those usually observed in European countries (29). The pH was around 7; the dry matter contents were 3.6% and 2.1% (wt/wt), respectively; the total Kjeldahl nitrogen contents were 3.1 and 3.6 g · liter^{-1}, respectively; and the ammonium contents were 80% and 45% of the total nitrogen, respectively. The concentrations of total volatile fatty acids were 6,888 mg · liter^{-1} in the storage tank and 331 g · liter^{-1} in the pond. As expected, the dry matter, total nitrogen, ammonia nitrogen, and volatile fatty acid contents were lower in the pond than in the storage tank. The differences resulted from the action of the press auger located between the outlet of the storage tank and the pond that received only the liquid phase of the manure. The distributions of the volatile fatty acids were the same in the two storage units: 60% acetic acid, 25% propionic acid, 5% butyric acid, and 10% isobutyric and isovaleric acids.

Bacterial 16S rRNA gene dynamics in pig slurry. The population dynamics of the pig slurry microbial community throughout the pig-rearing system were determined by 16S rRNA gene-targeted PCR-SSCP analysis.

The SSCP profiles obtained for fresh slurry samples (less than 2 weeks of storage) collected from the indoor pit at three different times had 15 to 20 distinguishable peaks that clearly emerged from the background of subdominant bacterial diversity (Fig. 1) (21). The presence of several comigrating peaks in the profiles suggests that there were microbial populations that were present in all samples.

The bacterial community SSCP profiles obtained during several stages of the slurry management process, including pig slurry storage in the outdoor tank and pond and subsequent spreading on the field, are aligned in Fig. 2. The profile for the storage tank at zero time, when the tank was almost empty,

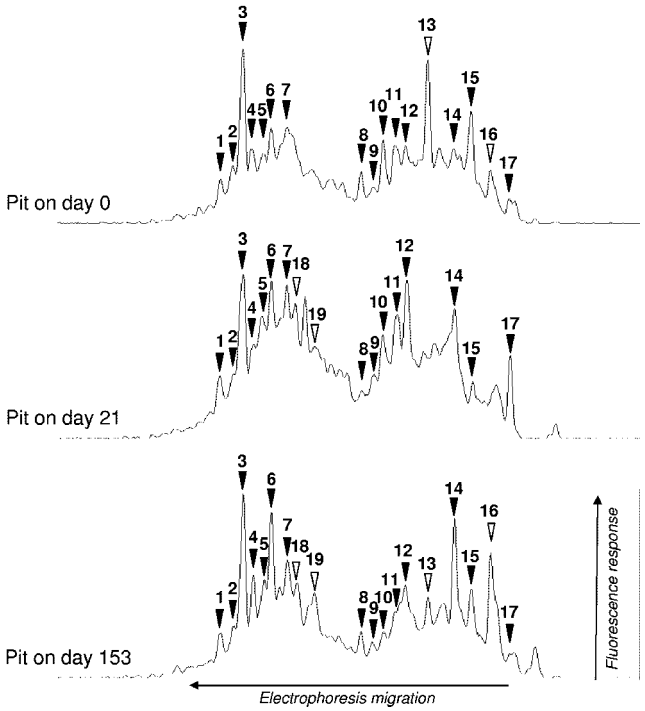

FIG. 1. Comparison of bacterial 16S rRNA gene-targeted PCR-SSCP profiles for fresh pig slurry (less than 2 weeks old) collected from the fattening building pit at three different times. SSCP electrophoresis was performed from right to left. The horizontal and vertical axes indicate time (number of scans) and detection of fluorescently labeled PCR products, respectively. The SSCP profiles for each sample are aligned for comparison. Peaks that comigrated in all profiles are indicated by solid arrowheads, while peaks 13, 16, 18, and 19, which comigrated in only two profiles, are indicated by open arrowheads. Peaks that were present in only one profile are not labeled.

shares several comigrating peaks with profiles obtained for pit fresh pig slurry, as shown in Fig. 1. However, the relative proportions of these peaks in the profile are different. After about 2 weeks of pig rearing and the addition of fresh pig slurry to the storage tank, the SSCP profile changed, and peak 3 became dominant in the community profile (day 21) (Fig. 2). Nevertheless, numerous small peaks remained in the profile and continued to comigrate with distinguishable peaks in the pit pig slurry SSCP profiles, suggesting that high microbial diversity persisted in the slurry during storage. After this, for the next 6 months of sampling, the SSCP profiles obtained for the storage tank samples remained very similar (Fig. 2) except for peak 7, whose relative proportion in the profiles seemed to increase.

The bacterial community SSCP profile for the pond sampled just before spreading indicates that there was a further apparent reduction in the bacterial diversity compared to the pig slurry profiles for the storage tank (Fig. 2). However, the majority of the peaks that were distinguishable in the pond SSCP profile comigrated with peaks present in the storage tank pig slurry profile. The analysis of four other pond samples obtained from April to June 2003 revealed that the bacterial community SSCP profiles for this structure remained the same (data not shown).

Finally, alignment of the pond SSCP profile with soil SSCP

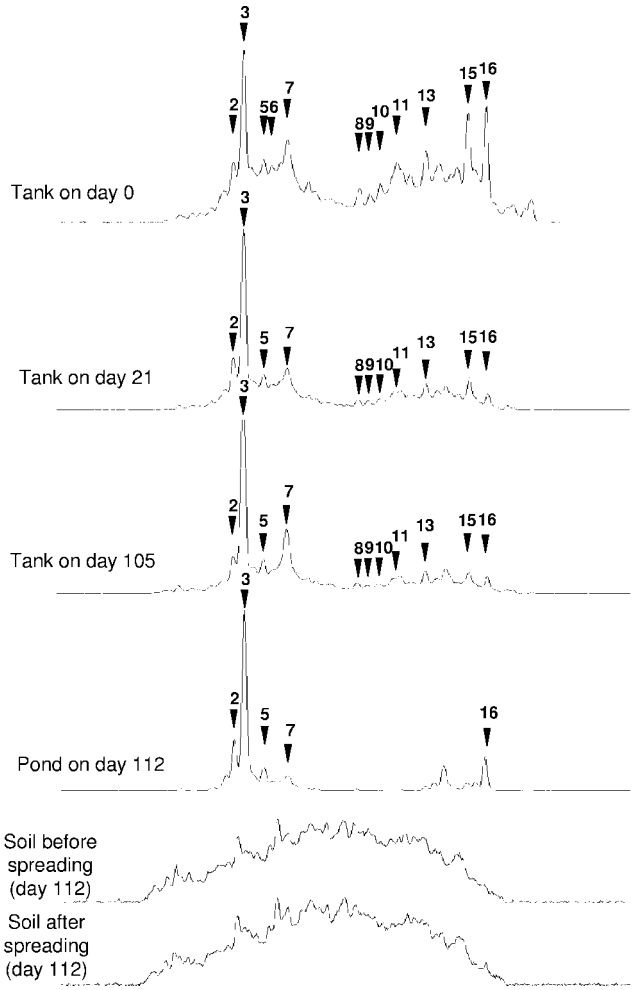

FIG. 2. Modification of SSCP profiles for the bacterial pig slurry community with time in the storage tank and at the time of spreading onto the field. SSCP profiles obtained for pig slurry collected at different times in the storage tank and at the time of spreading in the pond and onto the soil are aligned for comparison. Peaks present in Fig. 1 are indicated by arrowheads, and the same numbers are used.

profiles obtained both just before and after spreading of the liquid from the pond showed that none of the dominant peaks observed in the pond profile could be detected in soil after spreading. This remained true for soil samples collected 3, 7, 15, 28, 49, and 77 days after spreading (data not shown).

Monitoring of bacterial subgroups. The data presented above suggest that the bacterial community was stable throughout the pig slurry storage system. However, the use of general bacterial primers, the great diversity observed, and the presence of a very dominant peak (peak 3 in this study) may hide the variation of less dominant microbial groups (11). In order to better visualize the data, a nested PCR approach was used to artificially divide the bacterial SSCP profile. A set of primers targeting the *Eubacterium-Clostridium*, *Bacillus-Streptococcus-Lactobacillus*, and *Bacteroides-Prevotella* groups was used in combination with a general bacterial primer. According to data published previously (19, 31, 37, 43), these primers target more than 80% of the phylotypes observed in pig feces and pig slurry.

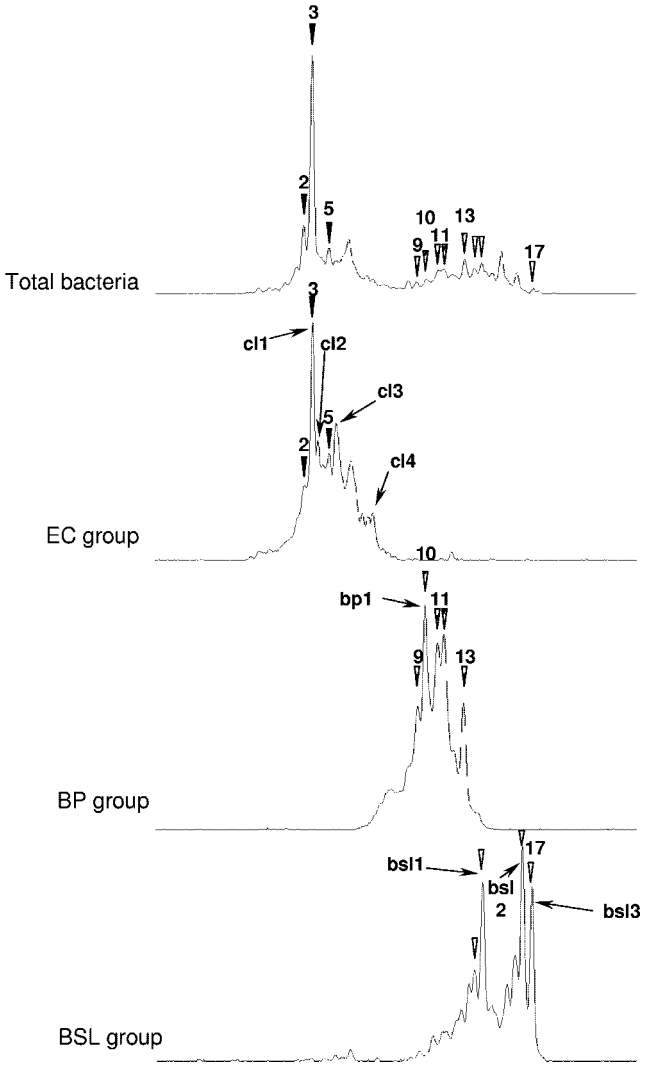

FIG. 3. Comparison of the bacterial SSCP profile with profiles obtained after nested PCR-SSCP targeting the *Clostridiaceae* (EC group), the BP group, and the BSL group. Amplification was performed with storage tank pig slurry samples obtained on day 21. The peak numbers are the same as the numbers in Fig. 1 and 2. Peaks that comigrated in the bacterial profile and group profiles are indicated by solid arrowheads for the EC group, by striped arrowheads for the BP group, and by open arrowheads for the BSL group. Peaks that could be identified are designated cl1 to cl4, bp1, and bsl1 to bsl3, as shown in Table 3.

Figure 3 shows an alignment of the SSCP profiles obtained for the same pig slurry sample for the bacterial community and for each targeted group. Each specific microbial group profile presents an individualized window of electrophoretic mobility in agreement with the expected size of its 16S rRNA V3 region that is centered around 179, 199, and 204 nucleotides for the EC, BP, and BSL groups, respectively. Use of this group-specific approach revealed diversity that was not detectable in the bacterial profiles. Nevertheless, several dominant peaks for each group comigrated with dominant or distinguishable peaks in the total *Bacteria* profile (Fig. 3). Thus, the very dominant peak 3 of the bacterial profile comigrated perfectly with the dominant peak of the EC profile.

TABLE 2. Phylogenetic affiliations of 16S rRNA gene sequences

Peak designation	Sequence length (bp)	Closest relative			
		Name (accession no.)	Affiliation group	% Similarity	Source
cl1	141	Clone THM-10 (AY147280)	*Clostridium botulinum*, cluster I	100	Thermophilic bioreactor treating piggery waste
cl2	141	Clone THM-8 (AY147278)	*Clostridium botulinum*, cluster Rumen	98	Thermophilic bioreactor treating piggery waste
cl3	144	Clone p-249-o5 (AF371793)	*Clostridium leptum*, cluster IV	95	Pig gastrointestinal tract
cl4	144	Clone P316 (AF261803)	*Clostridium leptum*, cluster IV	91	Swine manure storage pit
bp1	162	Clone p-987-s962-5 (AF371910)	*Porphyromonas*	97	Pig gastrointestinal tract
bsl1[a]	166	Clone SM8-19 (AY773140)	*Lactobacillus*	100	Swine manure
bsl2[a]	167	*Streptococcus alactolyticus* (AF201899)	*Streptococcus*	99	Swine feces
bsl3[a]	167	*Lactobacillus crispatus* (AF257097)	*Lactobacillus*	95	Human gut
c	119	*Methanogenium organophilum* (M59131)	*Methanomicrobiales*	97	Sediment
d	124	*Methanobrevibacter ruminantium* (AY196666)	*Methanobacteriales*	99	Bovine rumen
e	123	*Methanobrevibacter smithii* (AY196669)	*Methanobacteriales*	100	Intestinal tract of animal and sewage sludge

[a] Because of the short length of the 16S rRNA V3 region sequence, several other *Lactobacillus* (for peaks bsl1 and bsl3) or *Streptococcus* (for peak bsl2) sequences exhibited the same level of similarity as the closest relative shown.

This set of primers was used to monitor the population dynamics of each microbial group individually in the pig-rearing system and at the time of pig slurry spreading. As we observed for the bacterial community, the SSCP profiles obtained for the EC, BSL, and BP groups remained very similar over time for the pig slurry storage tank and to some degree for the pond. However, none of them was detected in soil after spreading (data not shown).

Identification of the persistent bacterial populations. We attempted to identify the dominant peaks observed for each microbial group by cloning and sequencing the corresponding 16S rRNA gene fragments, as described in Materials and Methods. Most of the dominant peaks in the EC and BSL profiles could be identified, while only the dominant peak in the BP profile was assigned (Fig. 3). The phylogenetic affiliations are shown in Table 2. The closest relatives of five of the eight sequences identified were sequences from uncultured bacteria that were obtained either from pig gastrointestinal tracts (peaks cl3 and bp1) (19), from a bioreactor treating piggery waste (peaks cl1 and cl2) (unpublished data), or from a pig slurry storage pit (peak cl4) (43).

Archaeal 16S rRNA gene dynamics. Monitoring of the archaeal community did not require a nested PCR and was performed by direct amplification of the archaeal 16S rRNA gene V3 region with domain-specific primers. In contrast to the results obtained for the bacterial groups, the diversity of the pig slurry archaeal community appeared to increase slowly during storage (Fig. 4). While the profiles at the beginning of the storage period contained only five dominant peaks (peaks a, b, c, d, and e), the profile obtained after 3 months of storage contained at least five other distinguishable peaks (peaks f, g, h, i, and j). At the same time, the dominance in the profile of peak d, identified as belonging to *Methanobrevibacter ruminantium* group I (Table 2), was slowly replaced by a dominant peak, peak b, that could not be identified.

Samples collected from the pond produced a simplified profile with reduced apparent diversity and the remarkable dominance of peak e, which was identified as *Methanobrevibacter smithii*. Judging from the peak alignment, this peak, as well as peak d (*M. ruminantium*) and peak c (*Methanogenium organophilum*), persisted from the storage tank to the pond. Detection of the archaeal community in soil before and after pig slurry spreading was attempted, but the 16S rRNA gene could not be amplified.

Fecal indicator dynamics in pig slurry. In order to obtain more insight into the behavior of the pig fecal microbial community, enterococci and *E. coli* were enumerated by cultural techniques during the pig slurry management process (Table 3). The concentrations of these microorganisms during the 6 months of storage were not significantly different for the building pit and the anaerobic storage tank, whereas the numbers of both fecal indicators in the pond decreased significantly ($P < 0.05$). In the soil there was no difference between samples collected before spreading and samples collected after spreading.

DISCUSSION

In this study, we used 16S rRNA gene-targeted PCR-SSCP analysis coupled with capillary electrophoresis and classical cultural techniques to monitor a pig slurry microbial community from an indoor pig-rearing pit to spreading on fields.

When molecular monitoring of the microbial community was used, very good reproducibility was observed for the SSCP profiles obtained from samples collected from the different matrices (pig slurry, pond, and soil) and at different times during the experiment over several months (Fig. 2). This can be explained by technical factors such as (i) the homogenization of the samples, (ii) the reproducibility of the sampling, (iii) the DNA extraction method used, and (iv) the use of capillary

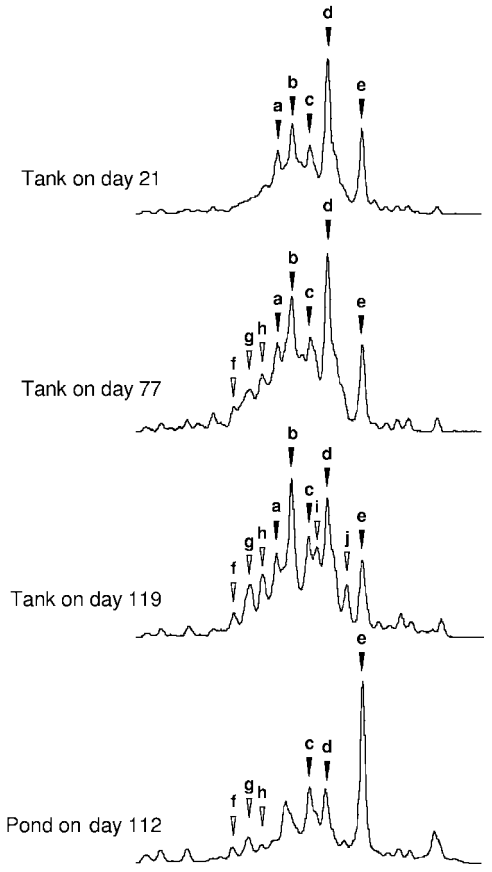

FIG. 4. *Archaea* pig slurry community dynamics during the pig slurry management process. The SSCP profiles for pig slurry samples collected at different times in the storage tank and in the pond are aligned for comparison. Peaks that dominated the profile at the beginning of pig slurry storage are indicated by solid arrowheads to facilitate comparison of the profiles. Peaks that appeared during storage are indicated by open arrowheads.

electrophoresis and automated DNA band detection, but also by the fact that the PCR-SSCP profiles revealed only the most dominant populations in the microbial community (the populations that were least prone to sampling bias). However, two limitations were clear when general bacterial primers were used (Fig. 2). The first limitation was the reduction in the apparent diversity of the profile in the presence of a very dominant population. The second limitation was the very peculiar shape of the SSCP soil profiles, which may be explained both by the high bacterial diversity present in the soil and by the absence of any very dominant microbial group (21). These two limitations could be partially overcome by the use of a nested PCR-SSCP approach. Focusing on specific microbial groups permitted visualization of subdominant populations that were not clearly visible in the total bacterial profile and allowed easier peak identification (Fig. 3). Each microbial group had a specific window of electrophoretic migration, confirming that SSCP electrophoresis separates single-stranded DNA fragments first according to size and second according to the secondary structure (18). The absence of detection of the spread microorganisms in soil, by both molecular and cultural techniques, was in all likelihood due to the high dilution rate for manure in soil because of the spreading itself and the concomitant dilution of the manure microorganisms in the soil microbial community. With slurry spread at a level of 170 kg hectare^{-1} of organic N and a density of 10^9 bacteria ml^{-1} of slurry, the number of microorganisms spread on the soil was about 10^{12} cells m^{-2}. If we assume that the density of microorganisms in soil is about 10^{10} bacteria g^{-1} (39) and the apparent density of soil is 1.5 kg liter^{-1}, the bacteria added with the slurry represented about 0.1% of the soil microflora. This value is too low to be detected by the techniques used in this study.

From a manure management perspective, SSCP monitoring of the dominant microbial populations showed that the pig slurry microbial community changed primarily during the first 2 to 3 weeks of anaerobic storage and then after the slurry was transferred to the pond. In contrast, the community remained very stable during passive anaerobic storage in both units. Cultural enumeration of fecal indicators revealed the same trend. The only significant decrease occurred between the storage tank and the pond. This stability of fecal indicator concentrations observed during pig slurry anaerobic storage contrasts with the decrease previously reported in batch studies (26, 27, 33). The difference may have been due to the constant addition of fresh feces to the slurry in real systems, which may logically lead to stabilization of the number of fecal indicators. Indeed, passive conditions that prevail in anaerobic storage tanks may not foster rapid change in the microbial community. According to this hypothesis, important microbial changes were observed in this study when the community was moved from the feces to the manure and then from the storage tank to the pond. Previous studies have demonstrated the stronger effects of drastic conditions like aeration or thermophilic treatment for modifying the pig slurry microbial community (17, 20, 26, 27).

Both methods showed that several microbial populations

TABLE 3. Average concentrations and ranges of concentrations of fecal indicators in the pig slurry management process

Parameter	Concn of fecal indicators (CFU g^{-1} [dry wt])									
	Building pit (day 0–day 189) (n = 4)[a]		Storage tank (day 21–day 189) (n = 8)		Pond (day 112–day 189) (n = 4)		Soil before spreading (day 84–day 112) (n = 2)		Soil after spreading (day 112–day 119) (n = 3)	
	Enterococci	*E. coli*	Enterococci	*E. coli*	Enterococci	*E. coli*	Enterococci	*E. coli*	Enterococci	*E. coli*
Mean	8.6×10^6	7.9×10^6	1.4×10^7	4.5×10^6	1.8×10^6	1.1×10^5				
Minimum	2.0×10^5	1.3×10^6	6.8×10^4	5.3×10^5	9.5×10^4	8.2×10^2				
Maximum	1.6×10^7	2.0×10^7	2.5×10^7	6.3×10^6	4.2×10^6	2.7×10^5	$\leq 2 \times 10^2$	≤ 12	$\leq 9 \times 10^2$	≤ 12

[a] n, number of samples.

remained present and dominant from rearing until the time of spreading. The fecal origin of the persistent microorganisms visualized by the SSCP profiles was confirmed by identification of the organisms. The eight bacterial SSCP peaks identified were closely related to microorganisms present in manure or feces (Table 3). Strongly dominant peak cl1 observed in stored pig slurry was identified as *Clostridium disporicum* (Y18176) and exhibited 99% sequence similarity for a 141-bp fragment. Although identification of microorganisms by using such a short sequence must be used carefully (35), it is interesting that this microorganism has been observed previously in a thermophilic aerobic digestor treating pig slurry (J. W. Lee, S. Y. Lee, and Y. K. Park, unpublished data) and as a persistent population in pig slurry after 7 weeks of aerated or unaerated storage (20). This group of *Clostridium* may be especially resistant to environmental changes.

The apparent stability of the pig slurry microbial community was tempered by the slow dynamics and increasing diversity observed for the archaeal community. Archaeal SSCP profiles showed that subdominant populations slowly adapted to their new environments in the storage tank and pond. All the peaks identified correspond to cultured H_2/CO_2- and/or formate-utilizing methanogens, but several subdominant peaks were not identified (Table 3). *M. ruminantium*, which was dominant in the community at the beginning of the storage stage, was replaced by *M. smithii* in the pond. Both of these species are commonly isolated from human or animal feces; however, the former requires more growth factors, including acetate (12). Differences between the concentration of organic matter (especially acetate) in the pig slurry in the storage tank and the concentration in the pond may explain this microbial shift. Peak c, which persisted until the end of storage, belongs to a group of microorganisms comprising *M. organophilum*, *Methanogenium frigidum*, and uncultured microorganisms observed in Antarctic sediment (32). The persistence of these archaeal groups is consistent with the methane production observed during pig slurry anaerobic storage (5 to 70 g $C\text{-}CH_4 \cdot m^{-3} \cdot day^{-1}$) (24). No acetotrophic methanogen was identified, despite the high acetate concentration in pig slurry. This absence has been noticed in two previous analyses of other pig slurry ecosystems (37, 44). However, we cannot rule out the possibility that such organisms were represented here by one of the subdominant unidentified peaks that appeared during the experiment.

The persistence of fecal microorganisms in human and animal organic wastes raises questions for all intensive manure management processes. This study showed that 16S rRNA gene-targeted molecular techniques can be used to detect microorganisms discharged into the environment by such processes. Further work should be done to identify these microorganisms and to determine their activity and quantity in effluent. For microorganisms that have not been cultured yet, this requires the use of quantitative molecular tools, such as real-time PCR (34).

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REFERENCES

1. **Altschul, S. F., T. L. Madden, A. A. Schaffer, J. Zhang, Z. Zhang, W. Miller, and D. J. Lipman.** 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. **25:**3389–3402.
2. **Bisaillon, J. G., R. Beaudet, M. Sylvestre, M. Ishaque, A. Morin, E. Di Franco, and A. M. Guérin.** 1984. Aspects microbiologiques du lisier de porc. Sci. Tech. Eau **17:**397–400.
3. **Braid, M. D., L. M. Daniels, and C. L. Kitts.** 2003. Removal of PCR inhibitors from soil DNA by chemical flocculation. J. Microbiol. Methods **52:**389–393.
4. **Brosius, J., T. J. Dull, D. Sleeter, and H. F. Noller.** 1981. Genes organisation and primary structure of a ribosomal DNA operon from *Escherichia coli*. J. Mol. Biol. **148:**107–127.
5. **Burton, C. H., and C. Turner.** 2003. Manure management. Treatment strategies for sustainable agriculture, 2nd ed. Silsoe Research Institute, Bedford, United Kingdom.
6. **Chackhiani, M., P. Dabert, T. Abzianidze, G. Partskhaladze, L. Tsiklauri, T. Dudauri, and J. J. Godon.** 2004. 16S rDNA characterisation of bacterial and archaeal communities during start-up of anaerobic thermophilic digestion of cattle manure. Bioresour. Technol. **93:**227–232.
7. **Chinivasagam, H. N., R. J. Thomas, K. Casey, E. McGahan, E. A. Gardner, M. Rafiee, and P. J. Blackall.** 2004. Microbiological status of piggery effluent from 13 piggeries in the southeast Queensland region of Australia. J. Appl. Microbiol. **97:**883–891.
8. **Cotta, M. A., T. R. Whitehead, and R. L. Zeltwanger.** 2003. Isolation, characterization and comparison of bacteria from swine faeces and manure storage pits. Environ. Microbiol. **5:**737–745.
9. **Delbes, C., R. Moletta, and J. J. Godon.** 2001. Bacterial and archaeal 16S rDNA and 16S rRNA dynamics during an acetate crisis in an anaerobic digestor ecosystem. FEMS Microbiol. Ecol. **35:**19–26.
10. **Delbes, C., R. Moletta, and J. J. Godon.** 2000. Monitoring of activity dynamics of an anaerobic digester bacterial community using 16S rRNA polymerase chain reaction–single-strand conformation polymorphism analysis. Environ. Microbiol. **2:**506–515.
11. **Duthoit, F., J. J. Godon, and M. C. Montel.** 2003. Bacterial community dynamics during production of registered designation of origin Salers cheese as evaluated by 16S rRNA gene single-strand conformation polymorphism analysis. Appl. Environ. Microbiol. **69:**3840–3848.
12. **Garcia, J. L., B. K. C. Patel, and B. Ollivier.** 2000. Taxonomic phylogenetic and ecological diversity of methanogenic Archaea. Anaerobe **6:**205–226.
13. **Godon, J. J., E. Zumstein, P. Dabert, F. Habouzit, and R. Moletta.** 1997. Molecular microbial diversity of an anaerobic digestor as determined by small-subunit rDNA sequence analysis. Appl. Environ. Microbiol. **63:**2802–2813.
14. **Hartung, J., and V. R. Phillips.** 1994. Control of gaseous emissions from livestock buildings and manure stores. J. Agric. Eng. Res. **57:**173–189.
15. **Hobson, P. N., and B. G. Shaw.** 1974. The bacterial population of piggery waste anaerobic digesters. Water Res. **8:**507–516.
16. **Iannoti, E. L., J. R. Fischer, and D. M. Sievers.** 1982. Characterization of bacteria from swine manure digester. Appl. Environ. Microbiol. **43:**136–143.
17. **Kumar, R., M. K. Gupta, and S. S. Kanwar.** 1999. Fate of bacterial pathogens in cattle dung slurry subjected to anaerobic digestion. World J. Microbiol. Biotechnol. **15:**335–338.
18. **Lee, D. H., Y. G. Zo, and S. J. Kim.** 1996. Nonradioactive method to study genetic profiles of natural bacterial communities by PCR–single-strand-conformation polymorphism. Appl. Environ. Microbiol. **62:**3112–3120.
19. **Leser, T. D., J. Z. Amenuvor, T. K. Jensen, R. H. Lindecrona, M. Boye, and K. Moller.** 2002. Culture-independent analysis of gut bacteria: the pig gastrointestinal tract microbiota revisited. Appl. Environ. Microbiol. **68:**673–690.
20. **Leung, K., and E. Topp.** 2001. Bacterial community dynamics in liquid swine manure during storage: molecular analysis using DGGE/PCR of 16S rDNA. FEMS Microbiol. Ecol. **38:**169–177.
21. **Loisel, P., J. Harmand, O. Zemb, E. Latrille, C. Lobry, J. P. Delgenès, and J. J. Godon.** 2006. Denaturing gradient electrophoresis (DGE) and single-strand conformation polymorphism (SSCP) molecular fingerprintings revisited by simulation and used as a tool to measure microbial diversity. Environ. Microbiol. **8:**720–731.
22. **Ludwig, W., O. Strunk, R. Westram, L. Richter, H. Meier, Yadhukumar, A. Buchner, T. Lai, S. Steppi, G. Jobb, W. Forster, I. Brettske, S. Gerber, A. W. Ginhart, O. Gross, S. Grumann, S. Hermann, R. Jost, A. Konig, T. Liss, R. Lussmann, M. May, B. Nonhoff, B. Reichel, R. Strehlow, A. Stamatakis, N. Stuckmann, A. Vilbig, M. Lenke, T. Ludwig, A. Bode, and K. H. Schleifer.** 2004. ARB: a software environment for sequence data. Nucleic Acids Res. **32:**1363–1371.
23. **Maidak, B. L., G. J. Olsen, N. Larsen, R. Overbeek, M. J. McCaughey, and C. R. Woese.** 1996. The Ribosomal Database Project (RDP). Nucleic Acids Res. **24:**82–85.

24. **Martinez, J., F. Guiziou, P. Peu, and V. Gueutier.** 2003. Influence of treatment techniques for pig slurry on methane emissions during subsequent storage. Biosyst. Eng. **85:**347–354.
25. **Martinez, J., and G. Le Bozec.** 2000. Pig manure and environmental concerns in Europe. Cah. Agric. **9:**181–190.
26. **Munch, B., H. Errebo-Larsen, and B. Aalbaek.** 1987. Experimental studies on the survival of pathogenic and indicator bacteria in aerated and non-aerated cattle and pig slurry. Biol. Wastes **22:**49–65.
27. **Olsen, J. E.** 1988. Studies on the reduction of pathogenic and indicator bacteria in liquid pig manure treated by sedimentation and anaerobic filter digestion for methane generation. Biol. Wastes **24:**17–26.
28. **Peters, S., S. Koschinsky, F. Schwieger, and C. C. Tebbe.** 2000. Succession of microbial communities during hot composting as detected by PCR–single-strand-conformation polymorphism-based genetic profiles of small-subunit rRNA genes. Appl. Environ. Microbiol. **66:**930–936.
29. **Peu, P., F. Beline, and J. Martinez.** 2004. Volatile fatty acids analysis from pig slurry using high-performance liquid chromatography. Int. J. Environ. Anal. Chem. **84:**1017–1022.
30. **Placha, I., J. Venglovsky, N. Sasakova, and I. F. Svoboda.** 2001. The effect of summer and winter seasons on the survival of *Salmonella typhimurium* and indicator micro-organisms during the storage of solid fraction of pig slurry. J. Appl. Microbiol. **91:**1036–1043.
31. **Pryde, S. E., A. J. Richardson, C. S. Stewart, and H. J. Flint.** 1999. Molecular analysis of the microbial diversity present in the colonic wall, colonic lumen, and cecal lumen of a pig. Appl. Environ. Microbiol. **65:**5372–5377.
32. **Purdy, K. J., D. B. Nedwell, and T. M. Embley.** 2003. Analysis of the sulfate-reducing bacterial and methanogenic archaeal populations in contrasting Antarctic sediments. Appl. Environ. Microbiol. **69:**3181–3191.
33. **Rivière, J., J. C. Subtil, and G. Catroux.** 1974. Etude de l'évolution physico-chimique et microbiologique du lisier de porcs pendant le stockage anaérobie. Ann. Agron. **25:**383–401.
34. **Rousselon, N., J. P. Delgenes, and J. J. Godon.** 2004. A new real time PCR (TaqMan PCR) system for detection of the 16S rDNA gene associated with fecal bacteria. J. Microbiol. Methods **59:**15–22.
35. **Schmalenberger, A., F. Schwieger, and C. C. Tebbe.** 2001. Effect of primers hybridizing to different evolutionarily conserved regions of the small-subunit rRNA gene in PCR-based microbial community analyses and genetic profiling. Appl. Environ. Microbiol. **67:**3557–3563.
36. **Schwieger, F., and C. C. Tebbe.** 1998. A new approach to utilize PCR–single-strand-conformation polymorphism for 16S rRNA gene-based microbial community analysis. Appl. Environ. Microbiol. **64:**4870–4876.
37. **Snell-Castro, R., J. J. Godon, J. P. Delgenes, and P. Dabert.** 2005. Characterisation of the microbial diversity in a pig manure storage pit using small subunit rDNA sequence analysis. FEMS Microbiol. Ecol. **52:**229–242.
38. **Spoelstra, S. F.** 1978. Enumeration and isolation of anaerobic microbiota of piggery waste. Appl. Environ. Microbiol. **35:**841–846.
39. **Torsvik, V., F. L. Daae, R. A. Sandaa, and L. Ovreas.** 1998. Novel techniques for analysing microbial diversity in natural and perturbed environments. J. Biotechnol. **64:**53–62.
40. **Van Dyke, M. I., and A. J. McCarthy.** 2002. Molecular biological detection and characterization of *Clostridium* populations in municipal landfill sites. Appl. Environ. Microbiol. **68:**2049–2053.
41. **Walter, J., C. Hertel, G. W. Tannock, C. M. Lis, K. Munro, and W. P. Hammes.** 2001. Detection of *Lactobacillus*, *Pediococcus*, *Leuconostoc*, and *Weissella* species in human feces by using group-specific PCR primers and denaturing gradient gel electrophoresis. Appl. Environ. Microbiol. **67:**2578–2585.
42. **Weisburg, W. G., S. M. Barns, D. A. Pelletier, and D. J. Lane.** 1991. 16S ribosomal DNA amplification for phylogenetic study. J. Bacteriol. **173:**697–703.
43. **Whitehead, T. R., and M. A. Cotta.** 2001. Characterisation and comparison of microbial populations in swine faeces and manure storage pits by 16S rDNA gene sequence analyses. Anaerobe **7:**181–187.
44. **Whitehead, T. R., and M. A. Cotta.** 1999. Phylogenetic diversity of methanogenic archaea in swine waste storage pits. FEMS Microbiol. Lett. **179:**223–226.
45. **Wood, J., K. P. Scott, G. Avgustin, C. J. Newbold, and H. J. Flint.** 1998. Estimation of the relative abundance of different *Bacteroides* and *Prevotella* ribotypes in gut samples by restriction enzyme profiling of PCR-amplified 16S rRNA gene sequences. Appl. Environ. Microbiol. **64:**3683–3689.
46. **Zhang, R. H., and D. L. Day.** 1996. Anaerobic decomposition of swine manure and ammonia generation in a deep pit. Trans. ASAE **39:**1811–1815.
47. **Zhu, J.** 2000. A review of microbiology in swine manure odor control. Agric. Ecosyst. Environ. **78:**93–106.
48. **Zhu, J., and L. D. Jacobson.** 1999. Correlating microbes to major odorous compounds in swine manure. J. Environ. Qual. **28:**737–744.
49. **Ziemer, C. J., M. A. Cotta, and T. R. Whitehead.** 2004. Application of group specific amplified rDNA restriction analysis to characterize swine fecal and manure storage pit samples. Anaerobe **10:**217–227.
50. **Zumstein, E., R. Moletta, and J. J. Godon.** 2000. Examination of two years of community dynamics in an anaerobic bioreactor using fluorescence polymerase chain reaction (PCR) single-strand conformation polymorphism analysis. Environ. Microbiol. **2:**69–78.