Characterization of the Bacterial Community in Stored Swine Waste Slurry Using 16S rDNA and PCR, with Emphasis on Odor Producing and Odor Destroying Bacteria

Final Report submitted to the
National Pork Producers Council (NPPC)

April 30, 1999

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CURRENT STATUS OF PROBLEM

Swine manure slurry is a very complex microbial ecosystem because the microbial community evolves from many different sources (e.g., intestinal tract, soil, water, feed); and the decomposition of the waste and ensuing odor problem involves the concerted activities of numerous bacterial species. While research has attempted to characterize the distinctive odor by identifying the odorous metabolites found in swine slurry, there is virtually no information on the bacterial species involved in odor production. Because of this lack of information, our efforts to remediate the odor problem are either unsuccessful or equivocal. Although production of these chemicals is the result of a dynamic microbiological activity, little is known about the specific types of microorganisms responsible for their production. Current cultivation techniques for isolating bacteria are estimated to recover only 1% of the total population in a complex ecosystem, thus other techniques must be used to characterize the microbial community, and identify the bacterial species responsible for producing the odors in swine slurry. The bacterial species responsible for destroying odorous metabolites in swine slurry are also unknown except a few bacterial species. The phylogenetic analysis of microbial diversity, using 16S rDNA and PCR, has been used to study complex ecosystems, such as activated sludge, marine sediment and gut. This technique has not been used to study the microbial diversity in swine waste slurry.

Odor emanating from anaerobic lagoons and swine production facilities has increased the tension among rural neighbors and among urban and rural residents. Storage of swine waste is associated with the production of a variety of odorous chemicals including ammonia, organic acids, alcohol, and sulfides. Much of this problem is due to the incomplete digestion process associated with anaerobic systems. In addition, production of odorous compounds within a confined swine facility can pose health problems to both the animals and human workers. Although production of these chemicals is the result of microbiological activity, little is known about the types of microorganisms responsible for their production. The results of this research are expected to provide new fundamental knowledge on the microbial population of stored swine manure slurry. Application of this information should result in the development of new approaches for the microbiological control of odor production.

INTRODUCTION

Environmental pollution by livestock waste is a major nationwide problem of the animal agriculture industry. Malodors emanating from livestock waste represent the major source of complaints against livestock farms. Anaerobic microbial decomposition of stored livestock waste appears to be the source of the more objectionable odors. More than160 odorous compounds have been identified in livestock wastes, which contributes to the complexity of attempting to characterizing the odor. Any attempt at odor remediation must first characterize the microbial diversity responsible for the odor production in the stored livestock waste. Knowledge of microbial community structure is fundamental for the effective management of both engineered and natural biological systems such as livestock waste. Amplified 16S ribosomal DNA gene sequences by PCR and restriction endonuclease analysis (ARDRA) is one technique which can be used to study diversity in a microbial community. The resulting restriction fragment patterns generated are used as a fingerprint for the identification of specific bacterial genome. This technique is based on the principle that the restriction sites on the RNA operon are conserved according to phylogenetic patterns. These patterns can be used for analyzing mixed bacterial populations. If the rDNA fingerprints for individual bacteria in a community are sufficiently different, then one can examine the amplified products for a series of distinct patterns resulting from the different population that make up the community. In general, ARDRA using the 16S rRNA genes will usually result in a pattern (3-5 bands per genome) when 4 base

site-specific restriction endonucleases are used Appreciable literature is available on the anaerobic microbial ecology of the swine intestinal tract, but this study deals primarily with the identification of types found in stored swine manure slurry. Little is known, however, regarding the role of these microorganisms and the interaction of these microbes in the production of odor. The knowledge gained from this research will be used to develop novel methods for potentially targeting specific odor-causing bacteria and manipulating their activities to reduce odor and/or ammonia production.

OBJECTIVE

The broad objective of this research is to characterize the population of microorganisms present in stored swine manure slurry. More specifically, the research is aimed at uncovering the underlying microbiological basis for odor from swine waste. A primary focus is to identify the microorganisms present in swine manure slurry and determine their contributions to the production of odor causing compounds. The results of this research are expected to provide new approaches for the micro-biological control of odor production.

MATERIALS AND METHODS

Manure Collection and Storage

The fresh swine manure was collected from the Michigan State University Swine Research and Teaching Farm, East Lansing, MI. Swine manure slurry collected from a storage pit at the swine facility was used to characterize the bacterial community. Since the swine manure slurry was periodically transferred to an anaerobic lagoon in order to get a reasonably fresh sample, the manure slurry was collected from the pit when it was relatively empty. The collected manure slurry from the samples in the pit was composited as an average representation from three different sites. The samples were homogeneously mixed, and then stored at room temperature in pre-sterilized covered glass container four and/or eight weeks. The sample was stored at freezer (-20°C) to minimize any changes that might occur in its physiochemical and biological properties until DNA extraction.

PROCEDURES

Total Bacterial Genomic DNA Extraction

Ten g of swine manure slurry was weighed into a 50 ml polypropylene centrifuge tube, and 20 ml of digestion buffer added. The mixture was frozen at -20°C until ready to extract. Thawed suspension was held on ice until used. A lysozyme solution (50 mg/ml) was prepared and 2 ml lysozyme solution and 2.0 g PVPP (polyvinyl polypyrrolidone) were added, shaken 100 times by hand, and incubated at 4°C for 2 hours. The freeze/thaw procedure helps disrupt cells, and the lysozyme solution degrades the bacterial cell wall. PVPP adsorbs humic materials. Eighteen ml of lysis buffer is then added and inverted gently to mix. This suspension is incubated at 4°C overnight. Wash buffer is refrigerated at 4°C. The suspension is centrifuged at 2000xg for 5 min, the supernatant is decanted into 250 ml-centrifuge bottles and held on ice. Twelve ml wash buffer is then added to the pellet and resuspend by stirring gently with stirring rod. Invert tube three times and centrifuge as before, pooling the supernatant. Repeat the washing step once more and then discard the remaining pellet.

Purification of DNA Extracted From Swine Manure Slurry

Purification was achieved by CsCl-EtBr density centrifugation. The 7 ml of extracted DNA recovered in the supernatant above is transferred to a clean 15 ml polycarbonate centrifuge tube. The 7 g of finely ground cesium chloride (CsCl) was added and mixed by gentle inversion. After the CsCl was fully dissolved, 0.7 ml of 10 mg/ml ethidium bromide (EtBr) was added. The DNA was banded by density centrifugation. DNA band is visualized by illumination with long wave UV light, which causes the EtBr-DNA complex to fluoresce. The DNA is remove from the gradient with a 16-gauge needle and 5 ml syringe. EtBr was remove from DNA by multiple extractions with isopropanol until the aqueous lower phase was colorless and then extracted once more. DNA sample is diluted with 2 volumes of distilled water, followed by addition of twice the diluted volume for cold (20°C) 100% ethanol. DNA was precipitated at -20°C overnight. The DNA is then collected by centrifugation at 7000xg for 30 min at 4°C, dried in a hood, and then suspended in 400 μl of distilled water. The DNA solution was transferred to a 1.5 ml microcentrifuge tube to which 40 μl of 3M sodium acetate (pH 5.20) was added, and the DNA was reprecipitated by addition of 840 μl of cold ethanol, followed by incubation overnight at -20°C. The DNA pellet was collected by centrifugation for 15 min at 4°C in a microcentrifuge, dried in a hood, and suspended in a small volume (about 100 μl) of distilled water. The purity of the processed DNA was determined by measuring the A260/A280 on a spectrophotometer. A ratio value of 1.5-1.8 was acceptable. DNA recovered by this procedure was suitable for PCR amplification and range from 66 to 93 μg/ml. The quality of the DNA was checked by electrophoresis of a 3 μl aliquot on a 0.8% agarose gel. This DNA (50 ng) was used in a standard PCR mixture, with the addition of 0.2 μl of bovine serum albumin per 20 μl reaction mixture.

PCR Amplification

Primers used for the PCR experiments were synthesized by MSU macromolecule facility. The primers used for the amplification of the 16S rDNA were 8-27 forward primer 5' AGAGTTT-GATCMTGGCTCAG 3', where M/A and 1510-1492 reverse primer 5' RGYTACCTTGTTAC GACTT 3', where R=A+G, Y=C+T. PCR reaction mix is total 20 μl; 10.3 μl d. H2O; 2 μl 10X PCR buffer; 2 μl dNTP mix; 0.8 μl of 8-27 forward primer (13.8 pmol/ml); 0.8 μl 1510-1492 reverse primer (15.1 pmol/ml); 1.7 μL DNA (50 ng/ml); 0.4 μl Taq (5 unit/μl): 2 unit/reaction; BSA (bovine serum albumin); 2 μl (final diluted concentration of 4 mg/ml from 20 mg/ml stock). Amplification was performed with a DNA thermal cycler under the following temperature condition: (1) 95°C for 1 min; (2) annealing step at 58°C for 1 min; (3) extension step at 72°C for 3 min; (4) repeat step 1-3, 30 times; (5) final elongation, 72°C for 5 min. The PCR product is checked by running a 3 μl aliquot sample on a 0.8% agarose gel, staining with eithidium bromide and visualizing by UV trans-illumination. The single band was close to the 1636 bp band of the 1 kb ladder. All PCRs were carried out with Taq polymerase and buffer (Gibco) in a Perkin Elmer 2400 thermal cycler.

Cloning

The Original TA Cloning Kit with pCR™ II (Promega) provided one step cloning strategy for the direct insertion of polymerase chain reaction (PCR) products into a plasmid vector. The ligation and transformation was carried out according to the manufacturer's instructions.

PCR Amplification for Cloned Colony

Primers used for the PCR experiments were synthesized by MSU macromolecule facility. The primers used for the amplification of the plasmid were forward primer 5' CAGTCACGACGTTG-TAAAACGACGGC 3', and reverse primer 5' CAGGAAACAGCT ATGACCATG 3'. PCR reaction mix is a total 100 μl; reaction mixtures containing 1xPCR buffer, 1.5 mM $MgCl_2$, 200 μM each of nucleotide (Boehringer GmbH, Mannheim, Germany), 15 pmol of each primer. Temperature profile was as follows: initial denaturation at 94°C for 30 sec; 30 cycles of denaturation at 94°C for 30 sec; annealing at 60°C for 30 sec; extension at 72°C for 2 min; and final extension at 72°C for 7 min. The PCR product is checked by running a 3 μl aliquot sample on a 0.8% agarose gel, staining with eithidium bromide, and visualizing by UV transillumination. The single band was about 1500 bp band of the DNA ladder. All PCRs were carried out with Taq polymerase and buffer (Gibco) in a Perkin Elmer 2400 thermal cycler.

ARDRA

PCR products are digested with the tetrameric restriction endonucleases HhaI, HaeIII, and RsaI (Gibco), as recommended by the manufacturers. Each digestion reaction is done with one of those restriction endonuclease HhaI, HaeIII, or RsaI. The digested were resolved by electrophoresis with 7 cm-long 2.5% Metaphor agarose gels (Biozym, Hess, Oldendorf, Germany) in Tris-borate-EDTA at 117V for about 3 hours at cold room. A 100 bp ladder (Gibco BRL, Eggenstein, Germany) was run at both sides and in the central lane of each gel. Gels were stained with eithidium bromide, visualized by UV transillumination, photographed, and the images saved on a disk as TIFF files.

Computer-Assisted Analysis of rDNA Restriction Patterns

Gel images were digitized with a charge-couple device video camera (INTAS, Gottingen, Germany) and stored on a disk as TIFF files. These were converted, normalized with the above mentioned molecular size markers, and analyzed with GelCompar software (version 4.0: Applied Maths, Kortijk, Belgium).

STATISTICAL ANALYSIS
(Computer-assisted pattern analysis of molecular fingerprints and database construction)

Proximity Coefficients

The analysis of fingerprint patterns generally requires a simplification of the original data via the generation of a proximity matrix based on dissimilarity or similarity criteria. Such proximity matrices can be established using a wide array of coefficients. The band-based comparison method, using the similarity coefficient defined by Jaccard, takes into account only the presence (or absence) of a band or peak and its position (binary variables). The coefficient derived by Dice also uses the information provided by the band position, but adds more weight to matching of band to their absence.

Clustering Methods

Cluster analysis of a collection of fingerprint patterns can be achieved by different ways. The input into a clustering algorithm is a proximity or resemblance matrix, and generally the output is a dendrogram. The goal of cluster analysis is to form groups with highly similar fingerprints in such a way that the fingerprints

of different groups are as dissimilar as possible. The choice of a clustering method is not always obvious and depends on the nature of the original data and the purpose of the analysis. Several algorithms are available for hierarchical or divisive clustering analyses leading to the generation of dendrogram. For ARDRA and IGS RFLP analysis, a band-matching algorithm was selected to calculate pair-wise similarity matrices with the Dice coefficient. A band-matching position tolerance of 2.0% and minimal area 2.0% was chosen. To analyze PCR patterns, as well as combined 16S rDNA restriction patterns with fingerprints, similarity matrixes of whole densitometric curves of the gel tracks were calculated by using the pair-wise Person's product-moment correlation correlation coefficient ('r value), an approach that compares the whole densitometric curves of the fingerprints. Using range from 100 bp to 800 bp to compare for HhaI, HaeIII, and RsaI restrictions compared 16S rDNA RFLP patterns (Figs.1 and 2). Cluster analysis of similarity matrices was performed by the unweighted pair group method using arithmetic averages (UPGMA) (Fig. 3).

DNA Sequencing of Partial 16S rDNA Fragments

Plasmids were purified with Promega's Wizard PCR-Prep columns (Promega, Mannheim, Germany). DNA sequencing of partial 16S rDNA fragments was submitted to the MSU DNA sequencing facility. Partial 16S rDNA sequences were amplified with primers 11 forward and 529 reverse.

Phylogenetic Analysis

Phylogenetic analysis of the deduced 5' to 3' rRNA sequence is initiated with its submission to the "Similarity Rank" routine of the Ribosomal Database Project(RDP). For each of the 16S rDNA clonal sequences a query is made to the RDP by using the Similarity Rank analysis service and to GenCANS to suggest the closest relatives within the RDP small subunit prokaryote rRNA database.

RESULT AND DISCUSSION

The bacterial community structure of stored swine manure slurry recovered from the pit in the MSU swine production facility was analyzed by ARDRA. One hundred and seven clones including the 16S rRNA gene inserts were produced and analyzed. Computer analysis of the DNA fragment patterns produced by restriction endonuclease (RE) digestion of the 16S rRNA gene inserts of individual clones resulted in the production of a dendrogram. Dendrograms are used to measure the relatedness between the clones and are useful in selecting which clones need to be sequenced. Sequencing clones, which have identical RE digestion patterns, has showed the accuracy of this method. To date, sequencing data has confirmed that clones inserted with 16S rRNA genes with identical RE patterns do have the same DNA sequence. Computer analysis of the sequence data was performed via the ANGIS database at the University of Sydney through GenBank (a worldwide database of known DNA sequences). This analysis indicated that a number of bacterial clones could be from bacteria that are of importance in public health, and two clones closely aligned with the genus *Clostridium* were identified. Many clones were grouped within the genus *Alcaligenes spp.* and some clones were either *Clostridium spp.* or *Bacteroides spp.*, indicating the presence of bacteria in the stored swine manure slurry that could be very important in the production and destruction of odor and could also represent a possible public health problem. Several dominant clones were sequenced to identify the abundance of their genera in stored swine slurry (Table 1).

Table 1. Abundance of bacterial genera in stored swine manure slurry.	
Genus	pproximate % abundance
Alcaligenes spp.	27
Bacteroides spp.	10
Eubacterium spp.	10
Clostridium spp.	0.2

The *Alcaligenes spp.* is a Gram-negative bacteria which occur e.g. in soil, water, and the intestinal tract of vertebrates and accounted for 27% of the clones. *Alcaligenes spp.* have been shown to degrade phenolic and indolic metabolites and their presence in the bacterial community structure would indicate a destruction of these compounds in the stored swine manure slurry and a transition to an improvement in odor. The *Bacteroides spp.* is a Gram-negative bacteria which occurs in the rumen, the mouth, and in the intestinal tract of man and other animals and accounts for 10% of the clones. *Bacteroides spp.* are highly proteolytic and their presence in the community would be indicative of ongoing odor production in the stored swine manure slurry. The *Eubacterium spp.* is a genus of Gram-positive, anaerobic, rod-shaped bacteria consisting of those species which are excluded from the genera *Actinomyces, Arachnia, Bifidobacterium, Lactobacillus,* and *Propionibacterium* on the basis of the nature of the products of fermentation. *Eubacterium spp.* typically form butyric and other fatty acids. Some species produce acetic and formic acids (and ethanol), and some do not form acids. *Eubacterium spp.* occurs in the human gut and in the rumen. The occurrence of *Eubacterium spp.* is also indicative of odor production in the stored swine manure slurry.

Clostridium spp., which accounted for only 0.2% of the clones, would appear to be a particularly good candidate as a problem group of organisms with respect to odor generation. During dry conditions they are capable of surviving for long periods of time as resistant spores, but when conditions become wet and anaerobic, growth is rapid and their metabolic processes generate large amounts of potentially odorous metabolites (e.g. p-cresol, phenol, indole, butyric acid). *Alcaligenes spp.* is considered the most abundant species in 8 weeks stored swine manure slurry, and may be actively involved in degrading odorous compound such as p-cresol during this stabilization period of storage. In contrast, *Clostridium spp.*, *Eubacterium spp.*, and *Bacteroides spp.* may be involved in the production of odorous compounds. The data suggest that the bacterial community structure of stored swine manure slurry consist of odor producing and odor destroying species which occur simultaneously with the odor destroying species increasing with longer storage time and more stabilization of the waste. Although the data fits with our concept of odor reduction of stored waste, we cannot definitively say those genera are the dominant bacterial species in stored swine manure slurry, because there is still some question about possible biases in the molecular approach of this evaluation. Consequently, the abundance of these genera is only an approximation of their dominance. Potential biases which could arise at different stages of the methodology are:

1. During extraction of the DNA, one assumes that substantially all the cells have lysed.
2. The PCR may introduce biases due to variations in primer binding or extraction efficiency.

3. It is known that some sequences will clone more readily than others and there are possible biases in the cloning step. These possible problems indicate that caution is still in order when one is interpreting data on the relative amounts of different clones in libraries, and one cannot assume that clones are found in proportion to their natural abundance. Examination of greater numbers of clones increases the level of confidence and the results appear to be realistic based on what is known about the bacterial community structure of swine manure.
4. There is also some concern about possible chimeras being formed during PCR amplification (check clone libraries). However, even if there are biases, they are probably quite unrelated to culturing biases. These approaches are yielding new information on the bacterial diversity present in stored swine waste slurry.

Future Research Objectives

Identification of the bacterial species responsible for odor production and destruction in swine manure slurry and their transition in the bacterial community with storage time, will allow researchers to target these species for either suppression or enhancement to reduce the odor problem. This will avoid the "shotgun" approach for odor reduction which indiscriminately kills the entire microbial population, including the beneficial bacterial species involved in the destruction of odorous metabolites. Bioremediation to control the odor problem would be preferable to chemical additives and other inhibitory compounds which could have a negative impact on the environment. Targeting a beneficial bacterial species for enhancement could speed up the decomposition process in swine waste slurry, thereby reducing the chance of volatile odors becoming a nuisance problem

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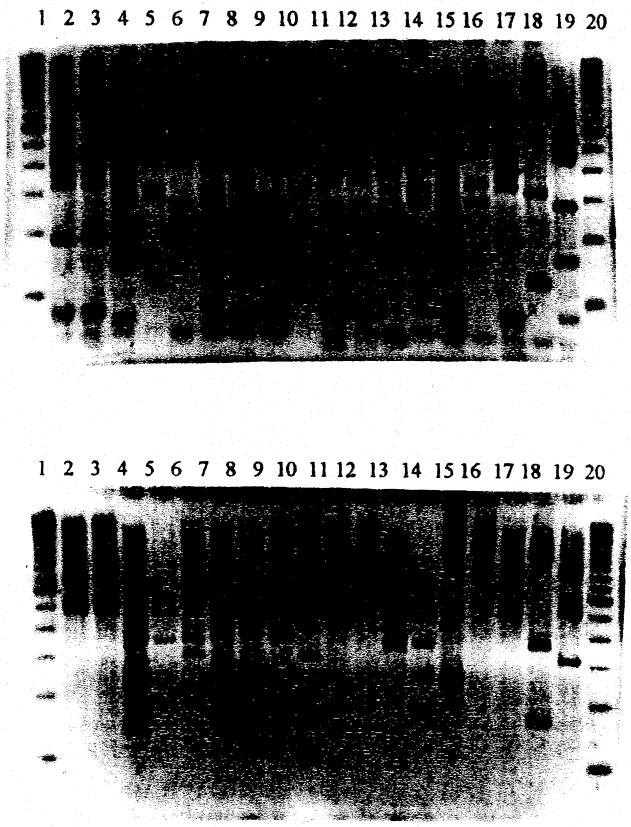
Fig. 1. Cloned 16S rRNA genes from stored swine manure slurry bacterial populations sorted and analyzed using restriction fragment length polymorphisms.



Lane 1, 10, and 20: 100 bp DNA ladder; Lane 2-9, 11-19: restriction digests of plasmids containing cloned 16S rRNA genes. The recombinant plasmids were digested with the restriction endonuclease HaeIII (above) and HhaI (below). The gel composition was 2.5% Metaphor agarose.

Fig. 2. ARDRA pattern: Digested with RsaI restriction enzyme.

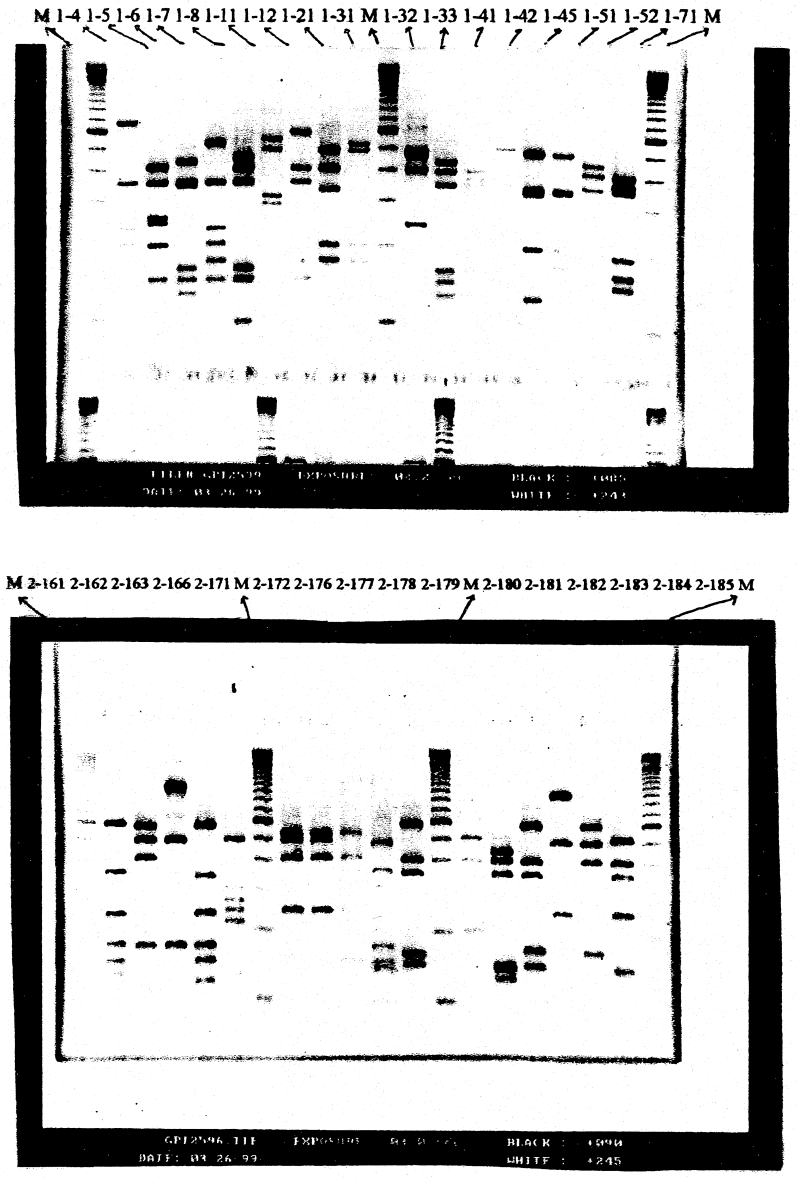


Fig. 3. Dice/UPGMA cluster analysis of combined HaeIII, HhaI, and RsaI restriction patterns of amplified 16S rDNA of stored swine manure slurry of the swine pit of MSU. The individual RFLPs are shown as bands defined on the actual restriction fragments.