MOLECULAR ANALYSIS OF THE MICROBIAL COMMUNITY DYNAMICS IN PIG SLURRY DURING STORAGE AND AFTER SOIL APPLICATION

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ABSTRACT

The evolution of the microbial community of a pig slurry was followed in a pig farm during 6 months. Sampling was carried out on all the different management steps of effluent: faeces, storage tank, lagoon of storage and soil before and after slurry spreading. Total DNA of these various samples were extracted and analyzed by PCR-SSCP, in particular for the archaea and bacteria domains and for specific bacterial groups. This study shows a relative stability in time of the dominant microbial populations present in the stored pig slurry. However, micro-organisms from the effluent could not be detected any longer in soil after spreading, using the SSCP technique.

The dominant *archaea* of the flora of faeces and pig slurry were identified as hydrogenotrophic achaea methanogens belonging to *Methanosphaera*, *Methanobrevibacter* and *Methanogenium*. No aceticlastic *archaea* methanogen was found, in spite of the strong acetate content measured in pig slurry. SSCP bacteria profiles obtained, reflect a large bacterial diversity where 9 dominant phylotypes could be characterized. Due to the complexity of the profiles obtained, three dominant bacterial groups were targeted: the *Clostridiacea*, the *Bacillus-Streptococcus-Lactobacillus* and the *Cytophaga-Flexibacter-Bacteroides*. Identification of dominant phylotypes was carried out for these 3 groups. The majority of identified phylotypes are close to uncultivated bacteria. With these results, identification of 5 of the 9 dominant bacterial phylotypes of pig slurry was carried out. The phylotype mostly represented in this ecosystem corresponds to an uncultured bacteria closely related to *Clostridium butyricum* and four other are close to uncultured *Prevotellaceae*, *Bacteroidaceae* and *Streptococcaceae*.

INTRODUCTION

Pig production in developed countries is often an intensive process that generates air pollution by emission of a great number of gas compounds such as ammonia volatilization, greenhouse gas and offensive odours, (Zhu, 2000). These problems are clearly associated to the microbial and physico-chemical transformation of pig slurry but only a few studies have investigated the overall composition and evolution of manure microbial communities during storage (Leung and Topp, 2001). The understanding of nuisances arising from pig slurry management will require the concomitant analysis of manure transformations and microbial community evolution. However, classical microbial studies have revealed that only 10 to 20 % of total microscopic cell count in pig slurry are culturable on media (Cotta et al., 2003).

The present work consisted in the characterization of the dominant microbial communities of a pig slurry with a molecular typing technique using small-subunit rDNA analysis: PCR-SSCP (Polymerase Chain Reaction and Single Strand Conformation Polymorphism) (Delbès et al., 2000). This approach was carried out throughout the pig slurry management process at an intensive pig fattening farm.

MATERIALS AND METHODS

Sampling was carried out on a commercial pig farm, holding 220 sows plus finishers, that produces about 4500 m^3 of pig slurry per year. On this farm, pig slurry is stored in a large outside tank with a total capacity of 800 m^3. In order to follow-up the microbial community dynamics in the effluent, various samples were collected over the six months of experiment: faeces on the stalled floor, slurry in the storage tank and slurry in the lagoon. Samples were put into storage within 12h and total DNA was extracted according to Godon et al. (1997).

Analysis of slurry for total bacterial community was performed by PCR amplification of the variable V3 region of microbial 16S rDNA using bacterial and archaeal primers according to Delbès et al. (2000). PCR products were separated and visualized by SSCP capillary electrophoresis on an automatic sequencer (ABI 310, Genetic Analyzer, Applied Biosystems). Peak identification was done by V3 PCR product cloning, clone screening by SSCP and sequencing of the clones of interest as described by Delbès et al. (2000).

Study of specific phylogenetic groups was carried out by nested PCR. In a first stage, the 16S rDNA of the targeted bacterial groups was amplified using specific primers coupled to a universal primer (W02: GNTACCTTGTTACGACTT or W18: GAGTTTGATCMTGGCTCAG; Godon et *al*., 1997). The primer W108 targets 60% of the sequences belonging to the *Bacillus-Lactobacillus-Streptococcus* group (ATTYCACCGCTACACATG; Heilig et *al*., 2002), W109 targets 80 % of the known *Eubacterium* and *Clostridium* sequences (TTACTGGGTGTAAAGGG; Wood et *al*., 1998), W112 targets 85% of the *Cytophaga-Flexibacter-Bacteroides* group (TCACCGTTGCCGGCGTACTC; Van Dyke and McCarthy, 2002). These selected groups contain about 85 % of the published sequences from pig gastrointestinal tract and manure. PCR products from this first PCR were then diluted and used as a template for the PCR-SSCP carried out using the same primers and conditions described above.

RESULTS AND DISCUSSION

Dynamics of the microbial community of the stored pig slurry was followed from December 2002 to June 2003. An alignment of the SSCP electrophoregrams from 3 samples obtained using general bacterial and archaeal primer sets is presented in Figure 1.

The microbial community appears as a profile of peaks where each peak corresponds to a dominant microbial population of the ecosystem. Peaks migrating at the same position are assumed to correspond to the same species. Archaeal SSCP profiles are relatively stable during time, five prominent peaks dominate all profiles. Three dominant archaeal peaks, located on the right side of the pattern (black arrows, Figure 1), were identified as *Methanosphaera*, *Methanobrevibacter* and *Methanogenium*. This result highlight the fact that all *Archaea* identified in the pig slurry ecosystem are methanogenic. Furthermore, the observed Archaea are known to use carbon dioxide or methanol plus free hydrogen as substrates. No known aceticlastic archaea methanogen was found in spite of the high acetate content of the stored pig slurry. Soil archaeal population was prospected before and after pig slurry spreading however no 16S rDNA PCR amplification was observed.

Bacterial SSCP profiles were very complex, with at least 20 distinguishable peaks (Figure 1). Nine of them were well individualized and reflected pig slurry dominant bacterial phylotypes (gray arrows, Figure 1). This phenomenon is characteristic of ecosystems having a great diversity. The highest diversity observed corresponded to soil profiles (data not shown) where the bacterial community was distributed all along the profile without detectable majority peak.

Comparison of the SSCP profiles revealed an unexpected stability of the pig manure slurry microbial community during time within the storage pit. From December 2002 to March 2003, the profiles remained the same with no major apparent change in peak number and composition. In March, however, a set of new peaks appeared in the middle of the profiles (striped arrows). These new peaks were found to originate from animal feces and probably result from a change in animal diet (Figure 1: feces, April 2003).

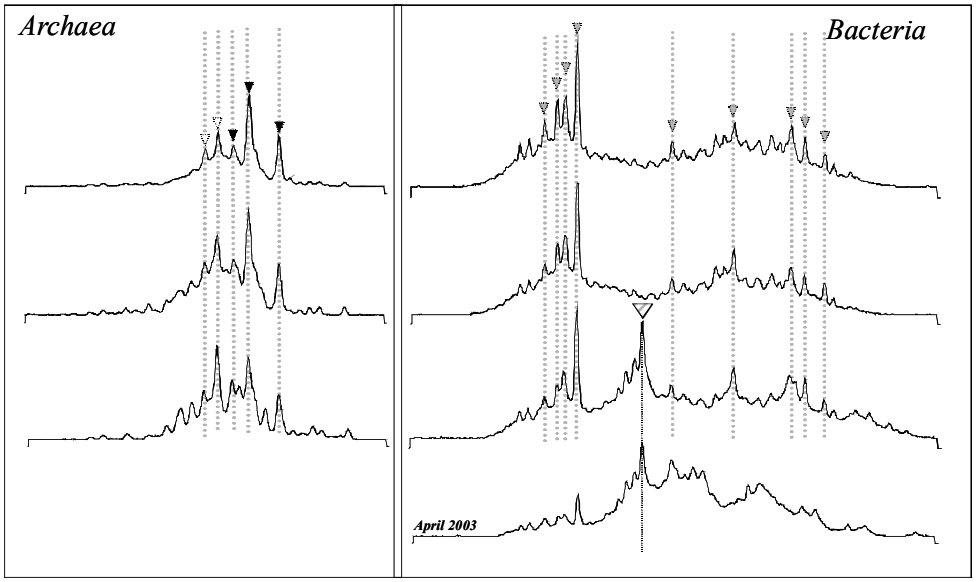

Figure 1. *SSCP profile evolution of a pig slurry bacterial and archaeal communities in the storage tank. In April 2003, only the fecal bacterial profile is shown.*

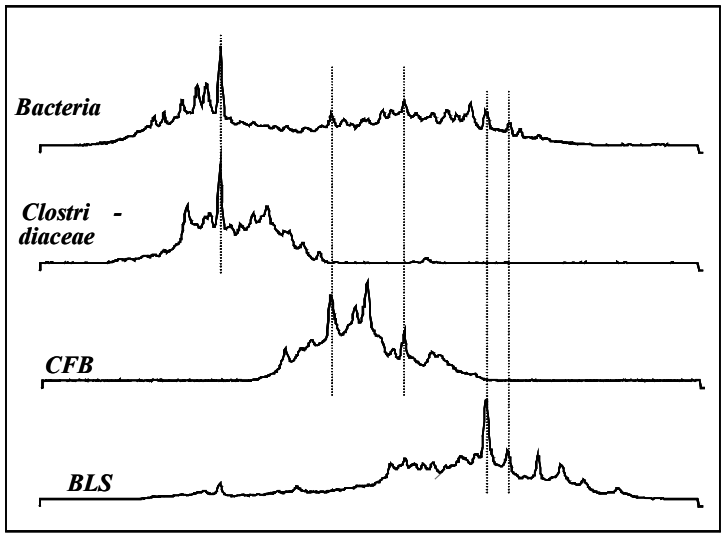

Figure 2. *"Separation" of the total Bacteria profile within the three dominant groups of the community by nested PCR-SSCP. BLS: Bacillus-Lactobacillus-Streptococcus; CFB: Cytophaga-Flexibacter-Bacteroides.*

SSCP profiles observed from lagoon samples showed a slow evolution of the community through time (data not shown). Finally, none of the peaks present in the profile from the lagoon pig slurry used for spreading were found in the soil profile after spreading (data not shown). This can be explained by the high dilution rate of manure microorganisms within soil at the time of spreading, which shows the detection limits using PCR-SSCP.

Since the PCR-SSCP allows the visualization of only dominant microbial populations from one sample, a simplification of the profiles was attempted using specific primers targeting the *Eubacterium* and *Clostridium* (*Clostridiacea*), the *Bacillus-Lactobacillus-Streptococcus* (BLS), and the *Cytophaga-Flexibacter-Bacteroides* (CFB). Figure 2 shows the resulting profiles for a same storage pit manure slurry sample. These profiles provide a better visualization of the dominant microorganisms within each group as well as facilitate their comparison with the bacterial profile.

Five dominant peaks found in group profiles co-migrate with five of the nine dominant bacterial peaks. The corresponding 16S rDNA fragments were sequenced. The dominant peak found in the stored pig slurry bacterial profile was identified as close to an uncultured bacteria related to *Clostridium butyricum* and isolated from a bioreactor treating piggery waste (Lee, et al. unpublished data). Two following peaks, co-migrating with CFB peaks, presented a high similarity with one sequence of uncultured *Bacteroidales* found in pig gastrointestinal gut (Leser et al., 2001). The dominant BSL peak presented a sequence strictly homologous with the one of an uncultured *Streptococcus* isolated from a swine manure storage pit (Cotta et al., 2003).

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