Bacterial community dynamics in liquid swine manure during storage: molecular analysis using DGGE/PCR of 16S rDNA

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Abstract

Denaturant gradient gel electrophoresis (DGGE) analysis of polymerase chain reaction-amplified DNA fragments prepared from extracted DNA using universal 16S rDNA primers was used to compare the composition of bacterial communities in liquid swine manure (LSM) during incubation in aerated and in non-aerated laboratory reactors. The LSM was initially dominated by 13 phylotypes whose identities were established by excising and cloning DGGE bands, and comparing the 16S rDNA sequences with those available in GenBank. With varying degrees of similarity, Clostridium butyricum, Clostridium disporicum, a Pedobacter sp., two Rhodanobacter sp., a spirochete, and seven uncultured eubacterial sequences were identified. The chemical composition, total microbial populations determined by direct microscopic count, and DGGE profiles of the LSM were stable during anaerobic storage for 7 weeks. However, the community composition of the LSM changed substantially with aeration, the DGGE bands in the original sample receding in intensity as the manure community became dominated by phylotypes most closely related to the aerobes Bacillus thuringiensis, Sphingobacterium mizutae, a 'Sphingobacterium-like' bacterium, and a Paenibacillus sp. When continuously aerated, the pH rose from 8 to 9.5, the ammonium-N content decreased, populations of culturable aerobes increased 150-fold, and total bacterial populations remained stable. DGGE analysis of manure fractionated into planktonic and biofilm (flocs and aggregates) communities suggested that Clostridium populations were stable in biofilms during aeration, whereas the aerobes that ultimately dominated the LSM community were primarily in the planktonic phase. © 2001 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

1. Introduction

Animal manures represent an important and valuable source of nutrients for agricultural crop production. At the same time, public health and environmental concerns regarding objectionable odours, chemical contaminants and pathogens pose a challenge for the storage and safe handling of animal wastes [1–4]. Storage conditions which promote the destruction of these contaminants have been the subject of numerous investigations, such as, controlling the range of redox conditions from aeration to anaerobic digestion, increasing storage temperature, drying and ozonation [2,5–13]. It has long been recognised that sub-populations of the global microbial population in liquid swine manure (LSM) may be responsible for breaking down various organic compounds and/or involved in suppressing pathogenic organisms during storage of the manure. However, only a few studies have examined the impact of storage conditions on the global microbial composition of LSM. These studies have been generally limited to the use of culture-dependent methods [5,9,14,15]. Recently, a study of the bacterial 16S rDNA sequences extracted and cloned from a human fecal sample found only 24% of the phylotypes corresponded to known organisms [16]. Furthermore, a similar study of bacteria in the colonic and cecal lumens of a pig showed that 59% of the 16S rDNA cloned sequences have less than 95% similarity to sequences from cultivated organisms [17]. Clearly the present information on the swine manure bacterial populations based on cultivation approaches may misrepresent the true diversity of microbial populations in LSM. As part of a study on the impact of aeration on the composition of animal wastes, we undertook a culture-independent examination of the major bacterial constituents of LSM and how the microbial community in the manure changes during storage in aerated and unaerated reactors.

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2. Materials and methods

2.1. Aerobic and anaerobic manure reactors

LSM was collected from a 600-animal farrowing farm located in South Western Ontario, Canada. The LSM consists of urine and faecal material from sows, and was obtained from the barn’s manure collecting tank (pit-type storage), which holds the LSM for periods of up to several months until it can be applied to adjacent fields. The tank is buried beneath the barn and thus the LSM would be sheltered from temperature and moisture-content fluctuations typical of an open outdoor lagoon. The composition of the LSM is reported in Table 1.

750-ml portions of fresh manure were dispensed into 1-l mason jars which were then capped with a lid fitted with several ports. The reactors were incubated for 7 weeks at room temperature (22 ± 1°C), with continuous agitation by means of a magnetic stirrer. Humidified compressed air was bubbled into the aerated manure at a flow rate of 120 ml min⁻¹, whereas anaerobic reactors were left unsparged and hermetically sealed. The headspace pressure in the unsparged reactors was released periodically by inserting a syringe needle through a rubber septum in the lid. All treatments were conducted in triplicate.

2.2. Chemical analyses

Manure samples were analysed for chemical composition using standard methods (Table 1; A&L Canada Laboratories East, London, ON, Canada). The pH and redox potential of the manure were measured periodically during the incubations with an Accumet pH/voltmeter (Fisher Scientific, Nepean, ON, Canada) equipped with a combination pH electrode (Orion Research, Beverly, MA, USA) or a platinum redox electrode (Orion Research) (Table 2). Reported redox values are relative to the normal hydrogen electrode.

2.3. Microbiological enumerations

Ten-fold dilution serial dilutions of the manure samples were prepared in 0.1% sodium pyrophosphate (w/v of water) and 0.1-ml portions of the sample solutions were spread-plated on Iso-sensitest agar medium (Oxoid, Basingstoke, Hampshire, UK). Colony-forming units (cfu) on the Iso-sensitst agar were determined following 4 days of incubation at 30°C.

Direct bacterial counts were performed on 5-[4,6-dichlorotriazin-2-yl]aminofluorescein (DTAF)-stained (Sigma-Aldrich Canada, Oakville, ON, Canada) manure samples [18]. Samples were preserved in formaldehyde (3.7% final concentration, v/v), and diluted 50-fold with sterile 0.85% NaCl solution. Ten μl diluted suspension was smeared in a pre-etched area (1.77 cm²) on a RITE-ON microscope slide (Gold Seal Products, Highpark, IL, USA). The smears were air-dried, heat-fixed and stained with 0.02% DTAF. Bacterial counts were determined by epifluorescent microscope equipped with a fluorescein filter set.

2.4. DNA extraction

Nucleic acid extraction was performed using UltraClean soil DNA isolation kits (MoBio Laboratories, Solana Beach, CA, USA) according to the manufacturer’s instructions with the following modifications. One ml of manure sample was collected from each manure reactor and pelleted in a 1.5-ml micro-centrifuge tube at 16 000 g for 10 min. The supernatant was discarded and the cell pellet was resuspended in 50 μl sterile deionised water and transferred to the 2-ml bead solution tube of the UltraClean soil DNA isolation kit. The sample was mixed with an

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Table 1

<table>
<thead>
<tr>
<th>Chemical analysis of the manure at the start of the incubation, and following 7 weeks of incubation under aerated or unaerated conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Manure a</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>Manure</td>
</tr>
<tr>
<td>Dry matter (%)</td>
</tr>
<tr>
<td>Organic matter (%)</td>
</tr>
<tr>
<td>Mineral N (%)</td>
</tr>
<tr>
<td>NH₄-N (%)</td>
</tr>
</tbody>
</table>

aMeans of two replicates.
bOrganic matter is reported on a dry matter basis.
cMineral constituents are reported on a weight per volume basis.

Table 2

| Redox potentials and pH of aerated and anaerobic manure samples a |
|------------------|------------------|
| Days  | Redox potential (mV) | pH               |
|       | aerated           | anaerobic        | aerated           | anaerobic        |
| 0     | −191 (± 2)        | −187 (± 1)       | 8.01 (± 0.07)     | 8.01 (± 0.10)    |
| 2     | +170 (± 6)        | −162 (± 11)      | 9.25 (± 0.04)     | 8.10 (± 0.02)    |
| 7     | +209 (± 9)        | −153 (± 8)       | 9.48 (± 0.03)     | 8.06 (± 0.02)    |
| 14    | +246 (± 5)        | −153 (± 14)      | 9.59 (± 0.04)     | 8.11 (± 0.01)    |
| 21    | +256 (± 6)        | −128 (± 19)      | 9.44 (± 0.05)     | 8.11 (± 0.06)    |
| 35    | +271 (± 15)       | −123 (± 10)      | 9.32 (± 0.06)     | 8.17 (± 0.19)    |
| 49    | +287 (± 5)        | −177 (± 7)       | 9.53 (± 0.02)     | 8.11 (± 0.23)    |

aData are means ± S.D.; n = 3.
inhibitor removal solution provided by the kit, and agitated twice in a Bio 101 Savant Fast Prep FP120 bead beater at a setting of 5.5 for 30 s. The crude DNA extract was purified by passing through a spin-column containing a DNA-adsorbing silica matrix as described by the manufacturer. Purified DNA was eluted from the matrix into 35 μl sterile buffer solution provided by the DNA extraction kit.

2.6. Cloning of 16S rDNA fragments resolved by DGGE

PCR primers targeting the V3–V5 region of 16S rDNA gene of all bacteria at the nucleotide positions 341–357 and 907–928 (based on the 16S rDNA of Escherichia coli, accession no. J01859) were used to amplify the 16S rDNA fragments of bacterial populations in the manure samples. The sequences of the forward and reverse primers were 5′-CCG CCG CCC CCT CCG GCG CCC GGC CCG GCC GTG CCG CCC CCC CGG CCT ACCT ACT GGA GGC AGC AG-3′ (GC clamp underlined) and 5′-CCG TCA ATT CCT TTG AGT TT-3′, respectively [19]. A touch-down PCR program was performed on a 50-μl PCR mixture containing 1.5 U of Taq DNA polymerase (Promega, Madison, WI, USA), 30 pmol of each primer, 1× PCR buffer, 1.5 mM MgCl₂, 200 μM dNTP and 2 μl of the 10-fold diluted purified manure DNA extract [20]. DGGE was performed with a DGene 16×16-cm 10% polyacrylamide gel (Bio-Rad, Hercules, CA, USA) maintained at 60°C in 7 l of Tris–acetate–EDTA (TAE) buffer (20 mM Tris-acetate, 0.5 M EDTA, pH 8.0). Gradient gels were prepared with 35 and 65% denaturant (100% denaturant defined as 7 M urea plus 40% v/v formamide). Between 0.5 and 1 μg of the amplified DNA was loaded per well and run at 100 V for 16 h. Gels were stained in 150 ml TAE buffer containing 15 μl 10 000× concentrated SYBR Green I (Molecular Probes, Eugene, OR, USA) for 1 h and destained in 350 ml TAE for 1 h. Images were captured using a Bio-Rad gel documentation system. Two approaches were used to ensure the correct designation of the DGGE bands in different samples. First, since the DGGE band pattern (such as bands A, H, J, K, B, C, D and E) of the initial LSM sample was highly consistent, it was used as a standard marker in different runs to facilitate gel-to-gel (or lane-to-lane) comparison. Second, some major DGGE bands (e.g. bands A, B, F, G and J) were sequenced twice under various occasions, such as under aerated and aggregate conditions, to confirm their phylogroups.

2.7. Sequence analysis

Three clones from each DGGE band were randomly chosen for sequencing analysis. If the sequence of the three clones from the DGGE band were not identical, three more clones from the same band were sequenced to estimate the number of 16S rDNA sequences co-migrating on the DGGE band. The cloned PCR fragments were sequenced by an ABI-Prism model 377 automatic sequencer (Perkin-Elmer, Forest City, CA, USA) using a T7 primer (5′-TAA TAC GAC TCA CTA TAG GG) targeting the T7 transcription initiation site of the pGEM-T vector. Sequence alignments were performed using the software program DNAMAN (version 4.0; Lynnon BioSoft, Vaudreuil, QC, Canada). Sequences were compared with the GenBank database by using the BLASTN facility of the National Center for Biotechnology (http://www2.ncbi.nlm.nih.gov/BLAST/) and were also tested for possible chimera formation with the CHECK_CHIPMERA program (http://www.cme.msu.edu/rdp/cgi/chimer.a.cgi?su = SSU).

2.8. Separation of biofilm and planktonic cells

Biofilm bacterial cells attached on the aggregate surfaces and clustered in large flocs were separated from planktonic bacterial cells by centrifugation. To optimise the separation of the biofilm and planktonic cells in the manure samples, the manure samples were centrifuged in a micro-centrifuge at 400×g for 30, 60 and 150 s. Microbial cells in the supernatant were stained with the LIVE/DEAD BacLight stain (Molecular Probes) and examined with an epifluorescent microscope for bacterial flocs and aggregates. Centrifugation at 400×g for 60 s was found to provide the best separation. The biofilm population in the samples were pelleted by centrifuging a 1-ml sample at 400×g for 60 s. The supernatant containing the planktonic microbial cells was centrifuged at 16 000×g for 10 min to recover the planktonic cells from the liquid phase. Samples containing the planktonic, biofilm and total microbial populations were stored at −20°C for DNA extraction.
2.9. Isolation of Bacillus and potential Sphingobacterium spp.

Because sequencing analysis of the two major DGGE bands (i.e. bands F and G) showed that the aerated LSM samples were dominated by the genera *Bacillus* and *Sphingobacterium*, the traditional cultivation approach was used to isolate potential *Bacillus* and *Sphingobacterium* at the highest dilution of the aerated LSM samples. The rDNAs of the isolates were sequenced and compared to the three dominant phylotypes in the aerated LSM identified by DGGE. A dilution series of the week 7 aerated manure samples were spread-plated on the Iso-Sensitest agar and *Bacillus*-selective agar (Oxoid). Ten potential *Sphingobacterium* (yellow-pigmented) colonies were isolated from the Iso-Sensitest agar and re-streaked on tryptic soy agar (TSA) plates. Ten presumptive *Bacillus* colonies were also isolated from the *Bacillus*-selective agar and re-streaked on TSA plates. Genomic DNAs were extracted from four randomly chosen presumptive *Bacillus* and *Sphingobacterium* spp. by the InstaGene Matrix DNA purification kit (Bio-Rad Laboratories) as described by the manufacturer. The DNA extract was used as the template in a PCR amplification as described earlier, except that the forward primer lacked the GC clamp. The PCR products were gel-purified as described earlier and the fragments were sequenced by an ABI-Prism model 377 automatic sequencer (Perkin-Elmer) using the DGGE forward primer (without GC clamp).

3. Results

3.1. Physico-chemical composition

Initially, the manure contained 0.6% dry matter, about a third of which was organic matter (i.e. lost upon ashing; Table 1). Almost all of the inorganic nitrogen was in the form of ammonium-N. The redox potential was about $-190 \, \text{mV}$, and the pH value about 8.0 (Table 2). These parameters remained unchanged throughout the experiment in the unaerated treatment (Tables 1 and 2). In contrast, aeration promoted rapid and significant changes in the manure. The redox potential increased to $+170 \, \text{mV}$ within 2 days, and up to $+287 \, \text{mV}$ by the end of the incubation. The pH increased to a value of $\text{pH} \, 9.25$ within 2 days, and remained at about $\text{pH} \, 9.5$ thereafter. By the end of the experiment the organic matter content had decreased somewhat, and almost all of the inorganic nitrogen and ammonium-nitrogen was lost. The aerated manure rapidly became dark brown, in contrast to the grey–black of the unaerated material. This colour difference was maintained throughout the incubation. In addition, after 2 days of aeration, the unpleasant odour of the manure disappeared in the aerated samples. The ammonia smell was detected transiently from the aerated sample in the first few days and disappeared afterward. On the other hand, the undesirable odours persisted in the anaerobic manure samples.

3.2. Dynamics of bacterial populations

Total bacterial populations enumerated by microscopic direct counts were stable at about $1 \times 10^{10}$ cells/ml in the manure regardless of the aeration treatment (Fig. 1). Culturable aerobic bacterial populations decreased somewhat from $1.2 \times 10^6$ to $3.0 \times 10^5 \, \text{cfu ml}^{-1}$ by the end of the experiment in the unaerated treatment. However, in the aerated reactors, culturable aerobic bacterial population increased about 150-fold from $7.3 \times 10^5$ to $1.1 \times 10^8 \, \text{cfu ml}^{-1}$ in 7 weeks, with most of the growth occurring in the first 7 days.

3.3. DGGE analysis

Background smearing in the DGGE profiles indicated the presence of a diverse community in the LSM (Fig. 2). However, the relative prominence of nine distinct bands indicated that the community was dominated by a relatively small number of phylotypes.

In the unaerated reactors no changes were observed in the DGGE profiles over the course of the experiment (Fig. 2). In contrast, three novel bands (F, G and I) emerged in the DGGE profiles of the aerated manure samples. Band F appeared at week 3 and increased in intensity to become one of the major bands by week 7. Band G appeared at week 5 and remained visible thereafter. Band I was a weak band visible at week 7. The appearance of these bands was accompanied by a decrease in the relative intensities of the nine DGGE bands dominating the community at the start of the incubation.

The relative composition of the planktonic and biofilm bacterial communities were different (Fig. 3). For example,
bands A and L were more prominent in the planktonic community, whereas bands C and B were more prominent in the biofilm community. The response of these communities to aeration was distinct. Following 7 weeks of incubation, the biofilm community was dominated by six bands (H, I, J, F, K and P) with the other initial bands receding in intensity. In contrast, the planktonic community at the end of the incubation was dominated by two bands (F and G) with other bands receding.

3.4. Sequence analysis

The major DGGE bands were cloned, sequenced and matched with the GenBank database (Table 3). Three to six clones were sequenced from each band. None of the cloned sequences were chimeric according to the CHECK_CHIMERA program. Eight bands (A, E, G, H, L, I, J and P) yielded a single 16S rDNA sequence, and the remaining bands (F, K, D, B and C) were composed of two co-migrating 16S rDNA fragments (Table 3). Bands in the initial LSM samples which corresponded to known organisms in the database included Clostridium butyricum (band H), Clostridium disporicum (J), a Spirochaeta sp. (L), a Pedobacter sp. (K), and two members of the genus Rhodanobacter (D). Percent similarities ranged from good (99%) to poor (89%).

4. Discussion

Previous studies have used various non-selective media to characterise bacteria in swine waste [9,14,15]. However, the disadvantages of cultivation approach are likely to result in an underestimate and/or bias in the diversity captured in these studies [14,15]. A few recent studies have used the culture-independent 16S rDNA-based PCR method to determine the diversity and dynamics of microorganisms in gastrointestinal tracts of pigs [17,22–24]. However, little is known about the culture-independent global microbial composition of LSM and the influence of oxygen status on the evolution of the microbial community during storage of LSM.

In this study, 13 major bacterial phylotypes were found in the initial manure samples (Table 3). Three phylotypes, identified as C. butyricum (clone H2), C. disporicum (clone J1), and a Rhodanobacter sp. (clone D2) showed ≥95% identity with sequences in the GenBank database. Other
phylogenetic groups identified with lower levels of identity included *Pedobacter* sp. (clone K1) and *Rhodanobacter lindanobacter* (clone D1). The clostridia are important constituents of the swine gut community and were probably excreted by the animals [4]. The other genera have not been reported to be enteric in these animals. *Pedobacter* is a recently described genus, proposed to be a member of the *Sphingobacteriaceae* fam. nov [25]. *Pedobacter* sp. are heparinase-producing obligate aerobes sharing the characteristic ability of growing at the expense of heparin [25]. Since heparin is produced in porcine intestinal mucosa (Sigma Life Sciences catalogue), it is possible to find heparin-degrading bacteria in the LSM. The *Rhodanobacter* sp. are Gram-negative aerobic bacteria in the γ-Proteobacteria group, distantly related to *Xanthomonas* and *Stenotrophomonas* [27]. Members of the *Rhodanobacter* sp. are soil bacteria but there is insufficient information known of this species to speculate on what its role in LSM might be.

The closest matching relatives to clones A3, K3, B1, B2, C1, C2 and E were either unculturable or unidentified eubacteria (Table 3). Interestingly, all these phylotypes were found in anaerobic environments, anaerobic vinasses reactors (GenBank accession numbers U81676, U81735 and U81735), anoxic rice paddy soil (AF229216), human gut (AF132261), bovine rumen (AF001778) or anaerobic marine sediments (4995896). Likewise, an uncultured spirochete most closely related to clone L1 is an organism isolated from a hydrogen sulfide-rich marine sediment (X93326). These results suggest that most of the phylotypes detected in the LSM are anaerobes. Absent from the sequenced phylotypes are some genera commonly found to be well represented in the pig gastrointestinal tract or faeces, notably *Lactobacillus*, *Streptococcus*, *Peptococcus*, *Peptostreptococcus*, *Propionibacterium*, *Ruminococcus* and *Bacteroides* [9,14,15,17,26]. It is highly probable that these organisms are in the LSM, but in low enough number that they are not prominent in the DGGE profile. Given that LSM consists of a mixture of urine and faeces and other detritus produced in the barn, that the collecting pit under the barn has a residence time of up to several months, and that it is continuously receiving fresh material, it is not surprising that the microbial composi-

### Table 3

Identity of phylotypes in DGGE profiles of aerated manure

<table>
<thead>
<tr>
<th>Band designation</th>
<th>Closest relative</th>
<th>% Similarity</th>
<th>Accession number</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Enriched upon aeration:</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>F</td>
<td><em>B. thuringiensis</em></td>
<td>94</td>
<td>457643</td>
</tr>
<tr>
<td>Clone F2</td>
<td><em>S. mitucae</em></td>
<td>90</td>
<td>887639</td>
</tr>
<tr>
<td>G</td>
<td><em>Sphingobacterium</em>-like bacterium</td>
<td>93</td>
<td>6729646</td>
</tr>
<tr>
<td>Clone G1</td>
<td><em>Paenibacillus</em> sp.</td>
<td>95</td>
<td>Y11583</td>
</tr>
<tr>
<td>I</td>
<td>uncultured bacterium</td>
<td>94</td>
<td>UBA400574</td>
</tr>
<tr>
<td><strong>Present in initial manure sample:</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>unidentified eubacterium</td>
<td>93</td>
<td>U81676</td>
</tr>
<tr>
<td>Clone A3</td>
<td>uncultured spirochete</td>
<td>90</td>
<td>AF211319</td>
</tr>
<tr>
<td>H</td>
<td><em>C. butyricum</em></td>
<td>99</td>
<td>X68178</td>
</tr>
<tr>
<td>Clone H2</td>
<td><em>C. disporicum</em></td>
<td>98</td>
<td>Y18176</td>
</tr>
<tr>
<td>J</td>
<td><em>Pedobacter</em> sp.</td>
<td>91</td>
<td>AB033630</td>
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<td>Clone K1</td>
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<tr>
<td>K</td>
<td>uncultured bacterium</td>
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<td>Clone B1</td>
<td>unidentified bacterium</td>
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<tr>
<td>B</td>
<td>uncultured rumen bacterium</td>
<td>88</td>
<td>AF001778</td>
</tr>
<tr>
<td>C</td>
<td><em>Rhodanobacter lindanoclasticus</em></td>
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</tr>
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<td>Clone D1</td>
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<td>AF250415</td>
</tr>
<tr>
<td>D</td>
<td>unidentified eubacterium</td>
<td>89</td>
<td>U81735</td>
</tr>
</tbody>
</table>

Indicated is the identity, % similarity, and accession number of the closest relative found in the GenBank database. Bands that were enriched during 7 weeks of continuous aeration as described in Section 2 are indicated. Band designations correspond to bands identified in Figs. 2 and 3.
tion of LSM is quite different from that excreted by the animal. Both the microbial and chemical composition of swine faeces is influenced by such factors as diet and animal age [24,28]. The composition of the LSM will additionally be influenced by farm management practices including feeding and watering regimen, temperature of the collecting pit, and the frequency of pit emptying [9]. In spite of this variability, we found DGGE profiles prepared from LSM obtained from the same collection pit at 3-month intervals to be remarkably similar, suggesting that our results are at least generally representative for this one farm (data not shown).

The chemical properties, redox potential, total number of bacteria determined by direct counting, the number of aerobes determined by plate count, and the relative composition of the LSM bacterial community determined by DGGE were remarkably constant in the anaerobic incubations. Because the manure-collecting tank at the farm holds the LSM for periods of up to several months before use, the liquid manure can be quite stabilised both chemically and biologically under anoxic conditions. Aeration caused a large increase in pH and redox potential, and significant change in chemical composition of the manure, evidenced by the loss of NH$_4$-N. Presumably volatile fatty acids were oxidised promoting alkalisation of the medium, and ammonium was either stripped out or fixed into growing bacteria. These profound changes in the aerated reactors did not result in a significant change in total bacterial population determined by direct counts. However, aeration promoted a 150-fold increase in aerobic populations detectable by plate count (representing only about 1% of the total population), and a significant shift in the community composition detected by DGGE. Most of the phylotypes originally present in the manure declined in relative intensity during the aerated incubation. Four phylotypes (clones F2, F3, G and I) were notably enriched by aeration. They were all related aerobic bacteria, the nearest relatives being B. thuringiensis (F2), S. mizutae (F4), a Sphingobacterium-like bacterium (G) and a Paenibacillus sp. (I) (Table 3). Paenibacillus sp., facultatively aerobic spore-formers, can grow at the expense of complex carbon sources such as whole rice, oats or wheat, and can produce amylolytic, phospholipolytic, lipolytic and proteolytic enzymes suggesting that they could utilise components of the swine diet [29,30]. Sphingobacterium sp., members of the Cytophaga–Flavobacterium–Bacteroides group, are capable of aerobic growth on a variety of carbohydrates, and are commonly isolated from soil and activated sludge [25]. Bacillus sp. are ubiquitous, and commonly but variably able to grow aerobically on complex substrates and lower molecular mass fatty acids including acetate and propionate [31].

Based on intensity in the DGGE gel, band F (clone F2-Bacillus, clone F4-Sphingobacterium) represented the most abundant phylotypes in the aerated manure at the end of the aerated incubation. Bacillus isolates randomly selected from agar medium for 16S rDNA sequencing analysis were 100% similar to B. thuringiensis or Bacillus sylvestris, but were more distantly related to clone F2, suggesting that the Bacillus detected in the DGGE analysis was not well-represented in the population recovered by plate counting. Furthermore, potential Sphingobacterium sp. isolates recovered from media did not correspond to the clone F4 band on the basis of 16S rDNA sequence. Taken together, although the number of clones sequenced was small, the results suggest that the plating methods used did not capture two of the dominant phylotypes in the DGGE analysis.

A comparison of the DGGE profiles prepared from total manure with profiles prepared from fractionated (biofilm and planktonic communities) manure indicates that changes due to aeration in the total community are reflective of changes occurring primarily in the planktonic population. At the end of the aerated incubation the planktonic community profile resembled the total community profile in the aerated manure. It exhibited decreases in almost all the initial phylotypes (bands A, H, L, J, K, B and D) and became significantly less complex. In contrast, the biofilm community responded quite differently to aeration than the planktonic community. Despite the disappearing of some of the initial major phylotypes (bands B, C and D), new phylotypes (band I, Paenibacillus; band F, Bacillus and Sphingomonas mizutae; band P, unculturable bacterium) started to establish and some initial biofilm phylotypes, such as C. butyricum (band H), C. disporicum (band J) and Pedobacter sp. (band K), remained dominant in the biofilm throughout the aerobic incubation. It is well-documented that matrix-embedded bacterial cells are more resistant to environmental insults [32]. Oxygen consumption by organisms at the surface of the biofilm, and slow diffusion of oxygen into the biofilm matrix can promote anaerobic microsites in aerated systems. Thus, denitrification and sulfate reduction can occur in sludge aggregates in thoroughly aerated water-treatment systems [19,33,34]. Presumably the higher relative abundance of the aerobes in the planktonic fraction is due to the availability of oxygen in the aqueous phase or at the surface of biofilms.

Clostridium sp. are active in stored LSM, fermenting lipids, sugars and amino acids to produce malodorous volatile organic acids, hydrogen sulfide and amines [4]. Clostridium perfringens is commonly used as an indicator of faecal pollution of water [35]. The DGGE profiles of total manure suggested that aeration promoted a decline in Clostridium populations. However, DGGE profiles prepared on fractionated samples suggest that this decline is illusory; that the mix of PCR products from the total manure sample was biased by the increase in planktonic template during the aerated incubation. Recognising that DGGE-PCR is not quantitative, the profiles do indicate that some organisms will preferentially partition into the biofilm. This result is significant for two reasons. Clearly
organisms embedded in the biofilm matrix will be less vulnerable to chemical or other treatment methods that could be used to reduce bacterial, especially pathogenic, populations in stored LSM. This phenomenon has been well-documented in numerous clinical, industrial and other applications [32]. Secondly, the potential for surface or groundwater contamination by indicator or pathogenic bacteria following manure application is likely to be affected by their distribution in the planktonic and biofilm phases. Adherence to flocs is a variable property, particularly influenced by cell surface hydrophobicity [36]. Bacteria which adhere to surfaces are less mobile in aquifer sediments than non-adherent bacteria [37]. It would therefore be of interest to determine the distribution of pathogenic and indicator bacteria in the biofilm and planktonic phases, how the spatial distribution may evolve during storage, and what influence this distribution may have on the persistence and transport potential of these bacteria following manuring of soils.

In summary, this is the first culture-independent study to examine the change of bacterial constituents in aerated LSM. The major bacterial phylotypes identified in our LSM samples were different from microbial species commonly found in the pig gastrointestinal tract or faeces by culture-dependent methods. Other than the two Clostridium spp., 11 out of 13 of the phylotypes identified in the LSM were not shown in any previous studies. The DGGE analysis also suggested that the aerated LSM was dominated by a Bacillus sp. and two Sphingobacterium spp. Although it was not clear if these phylotypes were involved in the biological and chemical changes of the aerated LSM, the findings provided valuable information to further study the roles of these micro-organisms in aerated LSM. Finally, we also showed that some bacterial phylotypes, such as the Clostridium spp., could be protected in the aggregates of LSM under constant aeration. This may have important implications on the persistence and transport of potential enteric pathogens in environments exposed to LSM.

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