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Bacterial community analysis of swine manure treated with autothermal thermophilic aerobic digestion

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Abstract Due to the enviornmental problems associated with disposal of livestock sludge, many stabilization studies emphasizing on the sludge volume reduction were performed. However, little is known about the microbial risk present in sludge and its stabilized products. This study microbiologically explored the effects of anaerobic lagoon fermentation (ALF) and autothermal thermophilic aerobic digestion (ATAD) on pathogen-related risk of raw swine manure by using culture-independent 16S rDNA cloning and sequencing methods. In raw swine manure, clones closely related to pathogens such as *Dialister pneumosintes*, *Erysipelothrix rhusiopathiae*, *Succinivibrioan dextrinosolvens*, and *Schineria* sp. were detected. Meanwhile, in the mesophilic ALF-treated swine manure, bacterial community clones closely related to pathogens such as *Schineria* sp. and *Succinivibrio dextrinosolvens* were still detected. Interestingly, the ATAD treatment resulted in no detection of clones closely related to pathogens in the stabilized thermophilic bacterial community, with the predominance of novel Clostridia class populations. These findings support the superiority of ATAD in selectively reducing potential human and animal pathogens compared to ALF, which is a typical manure stabilization method used in livestock farms.

Keywords Livestock sludge · Human and animal pathogens · Livestock sludge stabilization · Autothermal thermophilic aerobic digestion · Microbial community analysis

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Introduction

Livestock sludge pollutes soil, water, and air in close proximity to livestock farms. Finding an effective means of managing animal wastes has been a great challenge for environmental engineers and scientists (Pell 1997). Also, animals harbor many potential human pathogens. In a review of human infectious pathogens, 61% of 1,415 examined pathogen species were of a zoonotic origin (Taylor et al. 2001). Among them, *Escherichia coli* O157: H7, *Salmonella*, *Campylobacter*, and *Cryptosporidum* are readily transferred to humans causing enteric illnesses (Venglovsky et al. 2006). Traditionally, zoonotic pathogen infections were caused by consumption of contaminated animal products. However, due to the increasing practice of organic farming, in which livestock sludge is used as a fertilizing agent, now zoonotic pathogen infections are readily caused by consumption of contaminated organic products and polluted drinking water (Guan and Holley 2003; Franz and van Bruggen 2008).

In organic farming, stabilized (fermented/digested) livestock sludge is used as a fertilizing agent. Currently, a

typical method for livestock sludge stabilization is anaerobic lagoon fermentation (ALF) (UN Division of Sustainable Development 2000; US EPA 2003; Han et al. 2007). In this process, stabilization of the sludge takes at least 15 days, and operation procedure is difficult to control (Guan and Holley 2003). To circumvent these limitations, autothermal thermophilic aerobic digestion (ATAD) has been adapted as an alternative for stabilizing livestock sludge (Juteau et al. 2005). ATAD is a fast sludge stabilization process (approximately 3 days) that was originally used for the stabilization of municipal wastewater sludge (Matsch and Drnevich 1977; Jewell and Kabrick 1980; Kelly et al. 1993; Staton et al. 2001). Currently, only a limited number of studies have reported the effects of ATAD in the microbial community compositions of human pathogens and antibiotic-resistant bacteria in different types of sludge (US EPA 1990; Zabranska et al. 2003; Chénier and Juteau 2009a, b; Han et al. 2009).

It is well established that only a small portion of environmental microorganisms are culture dependent under laboratory conditions (Amann et al. 1995). However, a very limited number of culture-independent studies have been attempted to explore the effect of ATAD treatment on pathogen risk control although a few culture-independent studies were reported regarding antibiotic-resistant microbes in ATAD-treated sludge (Chénier and Juteau 2009a, b). Thus, culture-independent DNA-based analysis is needed for evaluating pathogen risk control in ATAD-treated livestock sludge. This study attempted to examine the possibility of pathogen-specific microbial risk control of swine manure by ATAD. For this purpose, we applied 16S rDNA gene cloning and sequencing methods. In addition, the pathogen-reducing effect of ATAD was compared with that of ALF, which is one of the most commonly used livestock sludge stabilization methods.

Materials and methods

Swine manure sample

The tested samples were obtained from Daun Swine Farm located in Seosan, Chungcheongnam-do, South Korea. The raw swine manure (LM) produced in the farm was collected into a field storage tank (7.0-m diameter, 10.0-m height) before being stabilized by either ALF or ATAD. From the field storage tank, 2 l of LM was collected for microbial community analysis. The swine manure was treated by ALF process in a field ALF tank (a batch type tank of 3.0-m width, 5.0-m length, and 5.0-m height with a solid retention time of 6 months). Operation temperature measured inside of the fermentation lagoon ranged between 20 and 40°C. After 6 months of the ALF operation, 2 l of ALF-treated swine manure (LF) was taken for microbial community analysis. In addition, the swine manure was treated by ATAD digestion process in a pilot-scale semi-batch reactor (2.5-m diameter and 4.0-m height), with agitation and aeration (85.2 m^3/h). The solid retention time of the ATAD process was 4 days. After a single day of the ATAD operation, the temperature inside the tank increased up to 65°C and was maintained between 60 and 65°C for the remaining operation period. After 4 days of the ATAD operation, efficient volatile suspended solid (VSS) removal (39.6% removal from 15,070±6,702 mg l VSS/l) was observed and 2 l of ATAD-treated swine manure (LA) was taken for microbial community analysis. More detailed information on the ATAD stabilization treatment is described elsewhere (Han et al. 2007, 2009), and its treatment outcomes are presented in Supplementary Table 1.

Bacterial 16S rDNA analysis

DNA was extracted from LM, LF, and LA using the BIO101 method with a Fast DNA SPIN Kit (Qbiogene Inc., Carlsbad, CA, USA). The extracts were spun down and concentrated using a bench-top microcentrifuge (6,000 rpm for 1 min). From each of the concentrated extracts, 0.5 ml (equivalent to 0.5 g VSS) of concentrate was sampled. The collected samples were added to 2 ml Lysing Matrix E containing 0.47 ml of sodium phosphate buffer and 0.122 ml of a lysis buffer (MT buffer). The mixtures were vortexed for 5 min to extract DNA from the cells. The rest of the DNA extraction steps were performed according to the manufacturer's protocol.

Bacterial 16S rDNA genes from the extracted DNA were amplified by polymerase chain reaction (PCR) using a thermal cycler (PTC1148 BMS, Seoul) and universal bacterial primers 27F [5′-AGAGTTTGATCATGGCTCAG-3′] and 1492R [5′-TACGGTTACCTTGTTACGACTT-3′] (Weisburg et al. 1991). The PCR reaction volume was 25 μl in total, and each reaction mixture contained 1× PCR buffer, 20 mM $MgCl_2$, 200 μM of each deoxynucleoside triphosphate (dNTPs), 1,000 pM of each primer (27F and 1492R), 0.025 U of *Taq* polymerase, 1 μl of DNA extracts (PCR template), and Millipore water (to give a total volume of 25 μl). All PCR reagents, except for the DNA extracts and sterile Millipore water, were provided with an Invitrogen PCR reagent kit (Invitrogen Inc., Carlsbad, CA, USA). PCR progressed with an initial denaturing step at 94 °C for 3 min, followed by 25 cycles of: (a) 94 °C for 1 min, (b) 50 °C for 25 s, and (c) 72 °C for 2 min. The extension of the last cycle occurred at 72 °C for 10 min. The PCR products were purified using Qiagen DNA purification spin columns (Qiagen Inc., Valencia, CA, USA). From the purified PCR products, 16S rDNA clone libraries were constructed for LM, LF, and LA using a TOPO TA cloning kit (Invitrogen). The constructed 16S rDNA clone libraries were then sequenced (Macrogen Inc., Seoul) and 37, 84, and 39 gene

sequences were obtained from the LM, LF, and LA clone libraries, respectively. The lengths of the obtained 16S rRNA gene sequence reads were approximately 800 bp.

Using the obtained 16S rDNA gene sequences, microbial population richness and diversity were explored in response to different stabilization treatments. For this, Chao1 index and Shannon index (H′) were adopted to analyze microbial richness and diversity, respectively. Chao1 is a good index to estimate population richness itself (Chao 1984) while Shannon index is good to estimate a combined diversity of population richness and evenness (Shannon and Weaver 1949). Prior to calculating the Chao1 and Shannon index values, programs provided by Ribosomal Database Project (RDP) II (Aligner Tool, Complete Linkage Clustering Tool, Classifier) were used to align, cluster, and classify the 16S rDNA gene sequences (http://rdp.cme.msu.edu/). Chao1 and Shannon index values were calculated using Shannon Index and Chao1 Estimator Tool from the RDP II. Using the Sequence Match software from the RDP II, we searched bacterial 16S rRNA genes close to our clone sequences. Among the matched bacteria, the closest known pathogen was selected for reference sequences for the following phylogenic analysis. To construct a phylogenetic tree, multiple alignments of our clone and reference sequences were conducted using MUSCLE software v4.0 (Edgar 2004, http://www.drive5.com/muscle/), and the aligned sequences were edited using BIOEDIT (http://www.mbio.ncsu.edu/BioEdit/bioedit.html). The lengths of the aligned and edited sequences were ~600 bp. The phylogenetic tree was constructed using seven, 11, and eight representative gene sequences from the LM, LF, and LA clone libraries, respectively. The phylogenetic tree was constructed using the MEGA4 program (Tamura et al. 2007). For the phylogenetic tree inference, the neighbor-joining algorithm was adapted and a bootstrapping test (1,000 replicates) was used.

Nucleotide sequence accession number

The GenBank accession number for the 16S rDNA gene sequences reported in this work are from (1) HM134993 to HM135030 for LM, (2) HM134954 to HM134992 for LA, and (3) HM069898 to HM 069897 and HM 135030 for LF.

Results

Microbial population richness and diversity

According to microbial population richness (Chao1 index) and diversity (Shannon index) result (Table 1), different stabilization methods affected richness and diversity. In the ALF-treated sample, population richness slightly decreased (4.2% reduction for Chao1) compared to that of LM, while the ATAD treatment resulted in a 65.8% decrease of Chao1 (Table 1). This indicates a significant reduction in population richness of swine manure by the ATAD treatment (Chao 1984). Compared to the Shannon index value of LM (H′=2.5), the ALF treatment resulted in 36.0% increase while the ATAD treatment resulted in 8.0% decrease in Shannon index. This indicates reduced microbial diversity in swine manure by ATAD treatment (Shannon and Weaver 1949). Because Chao1 and Shannon index reflects population richness itself and a combined effect of richness and evenness, respectively (Chao 1984; Shannon and Weaver 1949), these results suggests that the ATAD treatment resulted in reduction of population richness as well as evenness, while the ALF treatment maintained the population richness but increased its evenness.

Table 1 Microbial population richness (Chao1) and diversity [Shannon (H′)] analysis results for raw swine manure (LM), ALF-treated swine manure (LF), and ATAD-treated swine manure (LA)

Sample	N^a	Chao1	H′
LM	37	60.0	2.5
LF	84	57.5	3.4
LA	39	20.5	2.3

[a] N is the total number of clones in each clone library

Microbial community compositions and structure shifts

Initially, the microbial community of LM consisted of six bacteria classes (Fig. 1a), and “Minor” class group consists of Bacilli and Erysipelotrichi. After ALF treatment, microbial community compositions significantly changed at class level, and “Minor” class group consisted of more classes, i.e., Bacilli, Erysipelotrichi, and Mollicutes (Fig. 1b). Similarly, ATAD treatment also severely changed the microbial community compositions, and its “Minor” group consisted of only Bacilli class (Fig. 1c).

In the LM microbial community (Fig. 1a), major bacterial classes belong to unidentified Class (62.2%), Clostridia (16.2%), γ-Proteobacteria (10.8%), and Bacteroidia (5.4%), and two minor bacteria classes were Bacilli (2.7%) and Erysipelotrichi (2.7%). In the LF microbial community, major bacteria classes were unidentified Class (32.1%), γ-Proteobacteria (29.7%), Clostridia (19.0%), and Bacteroidia (9.5%), and three minor bacteria classes were Bacilli (2.4%), Erysipelotrichi (2.4%), and Mollicutes (4.8%). Finally, in the LA microbial community, major bacteria classes were Clostridia (61.5%), unidentified Class (25.6%), and Bacteroidia (10.3%), and minor bacteria class was Bacilli (2.6%). After the ATAD treatment, the γ-Proteobacteria class, which was under major bacteria class in the LM and LF microbial communities, had disappeared and Clostridia was identified

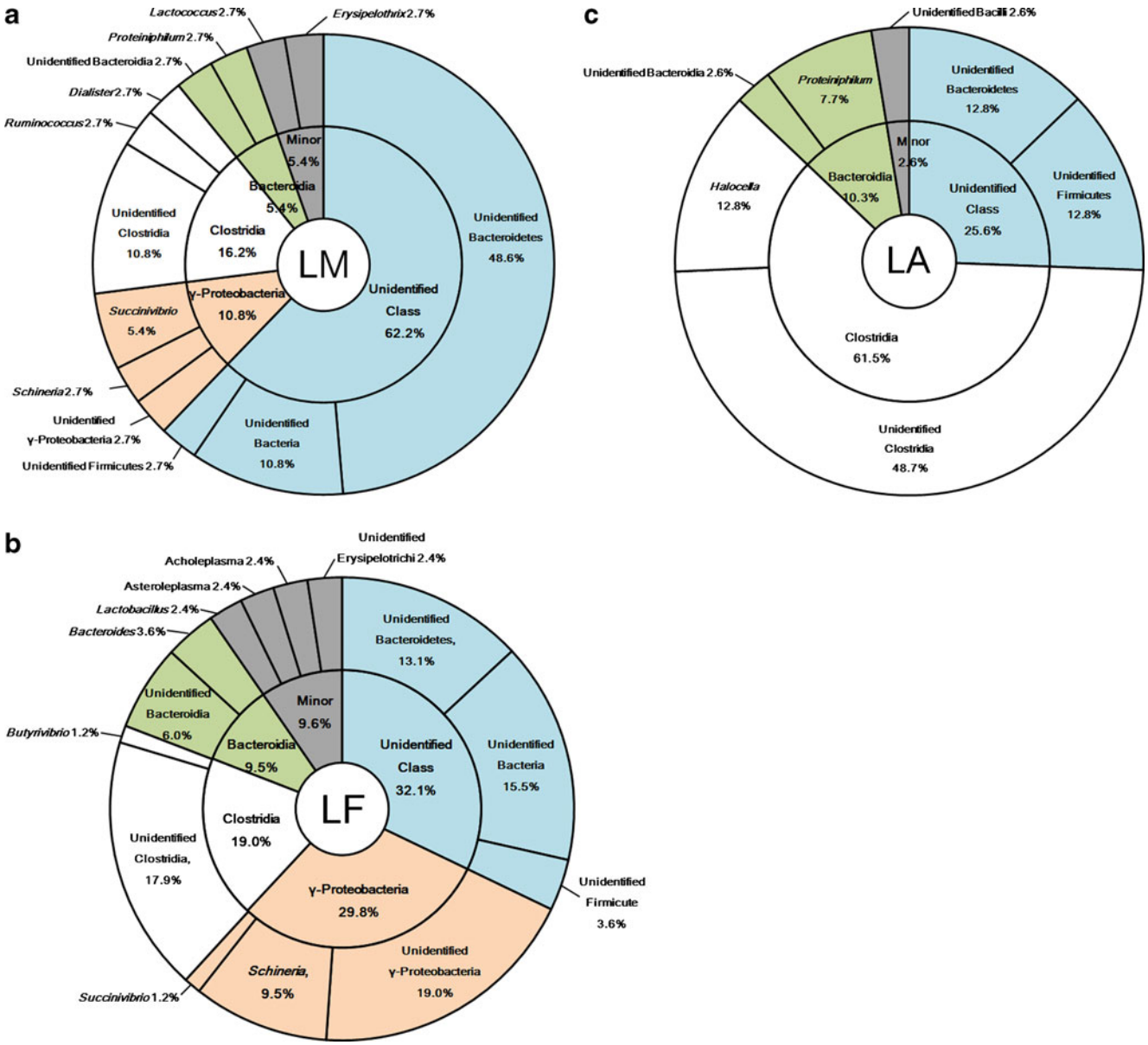

Fig. 1 Microbial community compositions at class level (*inner circle*) and at genus level (*outer circle*) of **a** raw swine manure (LM), **b** ALF-treated swine manure (LF), and **c** ATAD-treated swine manure (LA). The percentage indicates relative abundance in each clone library. *Minor* indicates class groups with relative abundances less than 5% in their communities. For LM, the minor classes were Bacilli (2.7%) and Erysipelotrichi (2.7%). For LF, the minor classes were Bacilli (2.4%), Erysipelotrichi (2.4%), and Mollicutes (4.8%). Bacilli (2.6%) was the minor class for LA

as the most dominant bacteria class in the LA microbial community. Meanwhile, Clostridia and Bacteroidia were identified as major bacteria class, and Bacilli were identified as minor bacteria class regardless of the tested microbial communities. In addition, between 25.6% and 62.2% of the clones were assigned to unidentified taxonomy, suggesting the presence of many novel bacteria in the swine manure and its stabilized products.

Figure 1 also presents results for the bacteria community structures at genus level [operational taxonomic units (OTUs) with 95% sequence similarity]. In the LM microbial community, major bacteria genus were unidentified Bacteroidetes (48.6%), unidentified Bacteria (10.8%), unidentified Clostridia (10.8%), and *Succinivibrio* (5.4%), and diverse minor bacteria genus (less than 5% relative abundance in each clone library 5%) were detected (Fig. 1a). In the LF community, major bacteria genus were unidentified γ-Proteobacteria (19.0%), unidentified Clostridia (17.9%), unidentified Bacteria (15.5%), unidentified Bacteroidetes (13.1%), *Schineria* (9.5%), and unidentified

Bacteroidia (6.0%), and diverse minor bacteria genus were detected (Fig. 1b). In the LA community, major bacteria genus were unidentified Clostridia (48.7%), *Halocella* (12.8%), unidentified Firmicutes (12.8%), unidentified Bacteroidetes (12.8%), and *Proteiniphilum* (7.7%), and two minor bacteria genus were detected (Fig. 1c). Among these major bacteria genus groups (greater than 5% relative abundance in each clone library), only *Halocella*, *Succinivibrio*, *Schineria*, and *Proteiniphilum* were identified at genus level while the other 95%-similarity-clustered OTUs were unidentified at genus level. This result indicates that most of the bacteria are novel in the tested samples.

Potential pathogen-related risk

To evaluate microbial risk of the samples, representative isolates from LM, LF, and LA 16S rDNA clone libraries were compared with type strains of known human and animal pathogens (Fig. 2). According to the phylogenetic analysis, the LM and LF isolates were clustered across a wide range of bacterial classes including Clostridia, Erysipelotrichi, γ-Proteobacteria, and Bacteroidia. However, the LA isolates were mostly clustered under Clostridia class, and only one isolate was under Bacilli class.

To compare the probability of human pathogen detection of the tested samples, the clones closely related to pathogens (sequence similarity cut-off >95%) were identified and their relative abundances (% of clone library) were estimated (Table 2). In the LM microbial community, clones close to potential human and animal pathogens such as *Dialister pneumosintes* (2.7%), *Erysipelothrix rhusiopathiae* (2.7%), *Schineria* sp. (2.7%), and *Succinivibrio dextrinosolvens* (5.4%) were detected. In the LF microbial community, clones close to *Schineria* sp. (9.5%) and *S. dextrinosolvens* (1.2%) were detected while none of clones were close to *D. pneumosintes* and *E. rhusiopathiae*. In the LA microbial community, interestingly, none of the clones were close to the pathogens. These results indicate that the LM community exhibited relatively high pathogen-related risk, and that the ATAD treatment resulted in improved reduction of pathogen-related risk compared to the AFL treatment.

Discussion

In this study, the pathogen-reducing effect of ATAD on raw swine manure was microbiologically evaluated using culture-independent 16S rDNA-based bacterial community analysis. The closest relatives of the LA isolates are *Halocella cellulolsilytica* DSM 7362 (Tsai et al. 1995) and *Bacillus thermocloacae* DSM 5250 (Rainey et al. 1994) which are non-pathogens. In contrast, many LM and LF isolates are close to known human pathogens such as *D. pneumosintes* (Willems and Collins 1995), *S. dextrinosolvens* (Southern 1975), *Schineria* sp. (Roudiere et al. 2007), and animal pathogen *E. rhusiopathiae* (Kiuchi et al. 2000). Although a few studies reported microbial diversity and community analysis results from ATAD-treated wastewater sludge and food waste slurry (Sonnleitner and Fiechter 1983; Staton et al. 2001; Ugwuanyi et al. 2008), there is a very limited number of studies for microbial community analysis of ATAD-treated animal waste (Lee et al. 2004; Juteau et al. 2005). In the other previous studies, Bacilli class populations were predominant in ATAD-stabilized microbial communities. However, in our ATAD-treated swine manure community, Clostridia class populations were the predominant group while Bacilli class was a minor group. According to the present work and other previous studies, Clostridia populations seem to be predominant in swine manure microbial communities (Peu et al. 2006; Snell-Castro et al. 2005) although the compositions of microbial communities differ in individual swine manure samples (Talbot et al. 2009). These suggest that the predominance of Clostridia might be a characteristic of swine manure microbial communities, and the predominant Clostridia populations could have grown under the conditions provided by ATAD treatment.

Among the Clostridia class populations in the ATAD-stabilized swine manure sample, 12.8% of its clone library was identified as *Halocella* genus population, and 48.7% was unidentified *Clostridium* genus populations. *Halocella cellulolytica* is the closest relative of the ATAD *Halocella* population. *H. cellulolytica* is a well-known thermophile with high cellulose-degrading activity (Simankova et al. 1993; Rainey et al. 1995). *Clostridium thermocellum*, *Clostridium straminisolvens*, and *Clostridium clariflavum* are known thermophiles to efficiently degrade variety of celluloses in biocompost (Izquierdo et al. 2010). However, the thermophilic cellulose-degrading *Clostridium* populations were not detected in our ATAD-treated swine manure sample. These suggest that *Halocella* and other non-*Clostridium* may have been involved in the efficient decomposition of swine manure biomass (39.6% VSS reduction) under the thermophilic conditions of the ATAD process. In addition, none of known endospore-forming Clostridia populations were detected in the ATAD-treated swine manure sample. This outcome may be beneficial in terms of pathogen risk control because endospore-forming Clostridia class populations are often animal pathogens such as *Clostridium perfringens* (Orsbur et al. 2010), *Clostridium botulinum* (Peck 2009), and *Clostridium sordellii* (Ramirez and Abel-Santos 2010). The predominant unidentified Clostridia class populations are novel since their 16S rDNA gene sequences are distant from known Clostridia class isolates (sequence similarity <90%). These findings led us to suggest that further isolation and

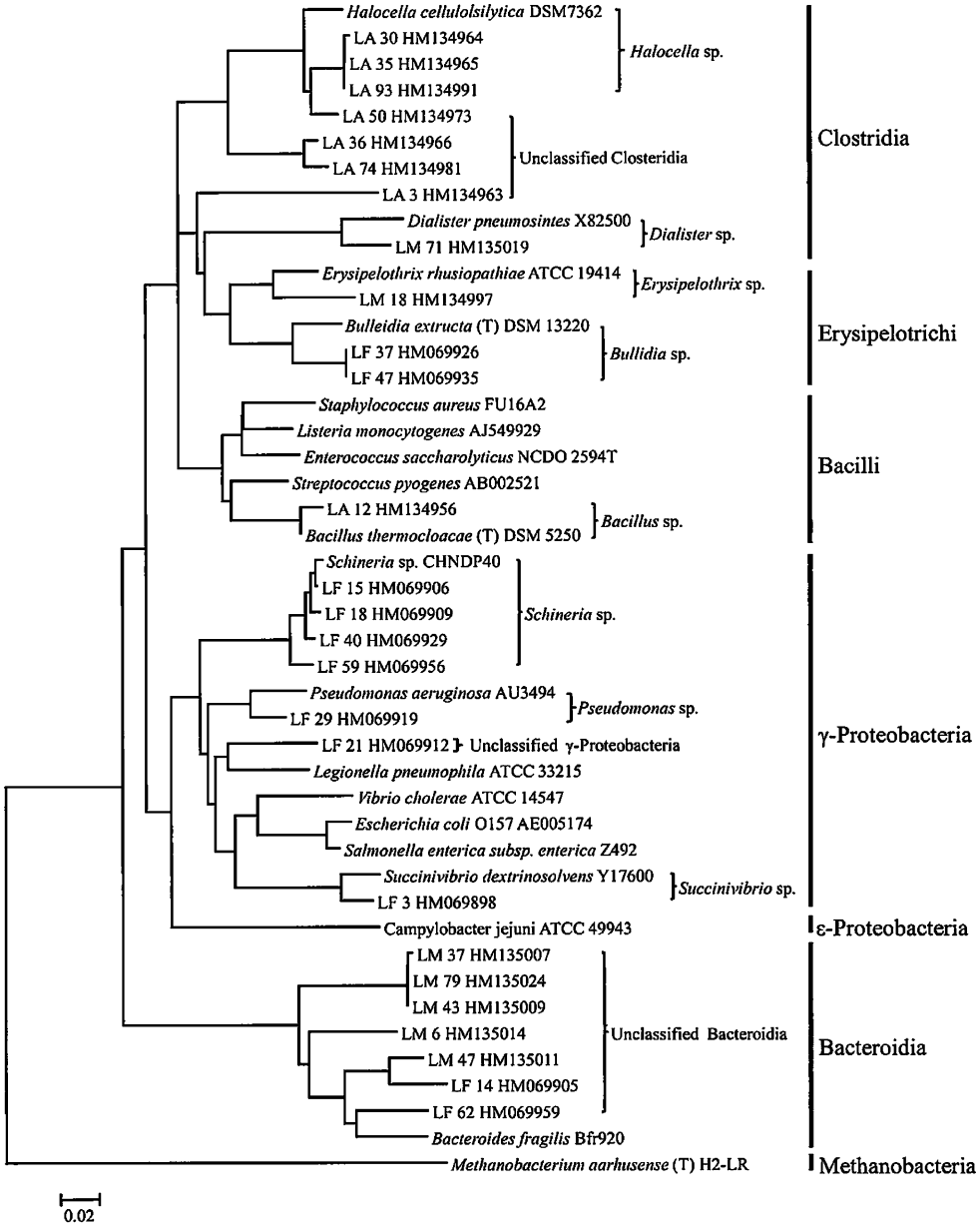

Fig. 2 Distribution of 16S rDNA gene sequences from clones of raw swine manure (LM), ALF-treated swine manure (LF), and ATAD-treated swine manure (LA) together with the reference sequences for the type stains of human pathogens. The *bars* on the *right side* of the figure indicate class-level identification. The *scale bar* on the *bottom left* indicates the distance of a 2% sequence divergence

Table 2 Relative abundances of potential human pathogens in each clone library [raw swine manure (LM); ALF-treated swine manure (LF); ATAD-treated swine manure (LA)]

Potential pathogen	LM (N^a=37)	LF (N=84)	LA (N=39)
Dialister pneumosintes	2.7%[b]	ND	ND
Erysipelothrix rhusiopathiae	2.7%	ND	ND
Schineria sp.	2.7%	9.5%	ND
Succinivibrio dextrinosolvens	5.4%	1.2%	ND

ND not detected

[a] N is the total number of clones in each clone library

[b] Unit: % of clone library

characterization is needed to understand the role of the novel Clostridia class populations in biomass decomposition and pathogen reduction in ATAD treatment.

Using the 16S rDNA-based bacterial community analysis, *Proteiniphilum*, which is an obligate anaerobe (Chen and Dong 2005), was detected in the ATAD-treated microbial community. The presence of an obligate anaerobe might be an indication of local anaerobic environment developed in the ATAD treatment. Due to the active decomposition of swine manure biomass by the ATAD treatment, oxygen demand may have been greater than the rate of aeration. In addition, incomplete mixing could have locally occurred in the pilot-scaled ATAD reactor. Insufficient aeration and/or incomplete mixing probably created local anaerobic or micro-aerobic environment in the ATAD reactor (Mavinic et al. 2001). This conclusion is consistent with Staton and his colleagues' (2001) previous report regarding molecular detection of anaerobic thermophiles in ATAD-treated wastewater sludge microbial communities.

The comparison with the ALF-treated community indicates that the different stabilization processes resulted in different outcomes of microbial richness/diversity and community compositions. Interestingly, ATAD treatment resulted in reduced pathogen detection in swine manure waste. Based upon the 16S rDNA analysis, 13.5% and 10.7% of the detected LM and LF bacterial populations were close to known human and animal pathogens. In contrast, none of the detected LA bacterial populations were phylogenetically close to known pathogens such as *D. pneumosintes* (Willems and Collins 1995), *E. rhusiopathiae* (Kiuchi et al. 2000), *Schineria* sp. (Roudiere et al. 2007), and *S. dextrinosolvens* (Southern 1975). This might be due to different temperature conditions. The temperature range for the ALF operation (20–40 °C) is mesophilic, under which human and animal pathogenic bacteria can grow (Watanabe et al. 1997). Meanwhile, most of zoonotic pathogenic bacteria might have been reduced under the ATAD-driven thermophilic (60–65 °C) condition. The conclusion of reduced pathogen risk by ATAD is consistent with the findings from other previous studies with ATAD-treated municipal wastewater sludge (Zabranska et al. 2003; US EPA 1990). While the other previous ATAD studies made the conclusion based upon the results of culture-dependent analysis, our current ATAD study could confirm their conclusion using culture-independent analysis.

Microbial risk may become higher when antibiotic-resistant microbes are co-present with pathogenic microbes because pathogenic microbes can gain antibiotic resistance via horizontal gene transfer from antibiotic-resistant microbes (Dzidic and Bedekovic 2003; Klare et al. 2003). Our previous study demonstrated that ATAD significantly reduced the culturable detection of multiple antibiotic resistant (MAR) bacteria in the same swine manure sample (Han et al. 2009) although antibiotic-resistance genes may have been present even in ATAD-stabilized swine manure (Chénier and Juteau 2009a, b). In contrast to the reduced MAR bacterial risk by the ATAD treatment, ALF resulted in increased probability of MAR bacteria detection in the same LF sample (Han et al. 2009). The co-presence of pathogenic and MAR bacteria in the swine manure and its ALF-stabilized product suggests extremely high microbial risk because it is difficult to treat infectious disease caused by MAR pathogens (Levy and Marshall 2004). Taken together with our previous work, the findings from this study suggest superior potential of ATAD treatment in controlling microbial risk compared to the conventional ALF treatment, which is typically used in livestock farms.

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