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Fate of tetracycline, sulfonamide and fluoroquinolone resistance genes and the changes in bacterial diversity during composting of swine manure

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ABSTRACT

This study monitored the abundance of antibiotic resistant genes (ARGs) and the bacterial diversity during composting of swine manure spiked with chlortetracycline, sulfadiazine and ciprofloxacin at two different levels and a control without antibiotics. Resistance genes of tetracycline (*tet*Q, *tet*W, *tet*C, *tet*G, *tet*Z and *tet*Y), sulfonamide (*sul*1, *sul*2, *dfr*A1 and *dfr*A7) and fluoroquinolone (*gyr*A and *par*C) represented 0.02–1.91%, 0.67–10.28% and 0.00005–0.0002%, respectively, of the total 16S rDNA copies in the initial composting mass. After 28–42 days of composting, these ARGs, except *par*C, were undetectable in the composting mass indicating that composting is a potential method of manure management. Polymerase chain reaction-denaturing gradient gel electrophoresis analysis of bacterial 16S rDNA of the composting mass indicated that the addition of antibiotics up to 100, 20 and 20 mg/kg of chlortetracycline, sulfadiazine and ciprofloxacin, respectively, elicited only a transient perturbation and the bacterial diversity was restored in due course of composting.

1. Introduction

Antibiotics are used in animal husbandries for both prophylactic and therapeutic purposes. However, 30–90% of the administered antibiotics are excreted through urine and feces as non-metabolized parent compounds and these non-metabolized antibiotics in the animal manure become a significant source of antibiotics and cause the development of antibiotic resistant microbes in the environment (Heuer et al., 2011). Consequently, there are concerns about the transfer of antibiotic resistance determinants to pathogens that may reduce the efficiency of antibiotic therapy to both human and animals (Barton, 2000). Development of resistance to antibiotics in microbes is a highly complex process and yet not completely understood even in clinical environments. But it was demonstrated that antibiotics even at sub-inhibitory concentrations affect cell functions and change the genetic expression of virulence factors or the transfer of antibiotic resistance (Salyers, 2002). The frequency of bacteria carrying antimicrobial resistance genes seems to be especially high for pigs as compared to cattle or sheep which correlates with the amounts of antibiotics used in the husbandry of these animal species (Enne et al., 2008; McKinney et al., 2010; Schwaiger et al., 2009). High usage of antibiotics put a pressure on the thriving microbes to evolve resistance for the specific environment. Besides, continuous prophylactic use of antibiotics would also facilitate the development of antibiotic resistant bacteria in animals that in turn act as a potential source of antibiotic resistance genes in the environment. Furthermore, animal manure promoted horizontal transfer of antibiotic resistance genes in soil were reported previously (Smalla et al., 2000). More details on the source of antibiotic resistance genes (ARGs) in soils and related impacts on the microbial dynamics can be found from the recent review by Heuer et al. (2011). Despite the neumerous reports on the presence and dissemination of manure-driven ARGs, their fate during the composting was never reported, although composting was demonstrated to reduce the levels of antibiotics significantly (Arikan et al., 2009; Dolliver et al., 2008; Hu et al., 2011; Selvam et al., 2012; Wu et al., 2011). As a promising bioremediation technology, composting has been applied to remove antibiotics from animal manures recently. During composting, the presence of a wide variety of complex organic compounds will encourage the development of a wide diversity and high population of microorganisms (Díaz et al., 1993). Usually, microorganisms dominating within the contaminated environment are those capable of utilizing and/or surviving toxic contamination. For their survival, they must possess appropriate mechanism for the tolerance; in case of antibiotics, presence of antibiotic resistance genes (ARGs) was implicated. On one hand removal of antibiotics from the animal manures is important; while, on the other hand the ARGs must also be eliminated to prevent their accumulation in soil.

DNA-based method, like real-time PCR (polymerase chain reaction), is being used increasingly in microbial ecology to quantify the functional gene markers within the environment (Smith and Osborn, 2009), owing to the feasibility of quantifying both culturable and non-culturable bacteria. There are studies concerning the

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abundance of certain ARGs in bovine feces (Alexander et al., 2011), groundwater near the concentrated animal feeding operations (Aminov et al., 2001,2002; Chee-Sanford et al., 2001) and swine manure (Agersø et al., 2006). Further, prevalence of *tet* and *erm* genes in swine manure compost were quantified in a couple of previous studies (Chen et al., 2007; Yu et al., 2005). The *tet* genes belonging to RPP (ribosomal protection protein) group were undetectable in two-thirds of the swine manure compost samples; whereas the *tet* genes belonging to 'efflux' group were reduced up to 6 logs (Yu et al., 2005); and *erm* gene abundances were reduced by up to 7.3 logs (Chen et al., 2007). These results indicate that the composting can reduce the ARGs significantly; however, the detailed information regarding the ARGs distribution during manure composting as well as the microbial diversity changes upon antibiotic presence is still lacking. Tetracyclines and sulfonamides were the most used antibiotics in high quantities in swine farms. Further, compared with cattle and poultry, swine manures were reported to have higher *tet* and *sul* resistance genes in the effluent as well as in the solid phase (McKinney et al., 2010) that requires suitable mitigation. Therefore, this study aimed at monitoring the selected ARGs responsible for the tetracyclines, sulfonamides and fluoroquinolones during composting of swine manure spiked with chlortetracycline (CTC), sulfadiazine (SDZ) and ciprofloxacin (CIP). Additionally, changes in the bacterial community profile with the aim to understand how antibiotics affect the bacterial diversity were investigated using PCR-DGGE (denaturing gradient gel electrophoresis).

2. Methods

2.1. Reactor and operation

The swine manure was collected from the farm in the new territories, Hong Kong and the selected physicochemical properties were as follows: pH 7.13, moisture content 74%, bulk density 1.04 t/m^3, total organic carbon 30.69%, total Kjeldahl nitrogen (TKN) 4.03%, organic matter 72.1% and carbon/nitrogen (C/N) ratio 7.6. The swine manure was spiked with SDZ, CTC and CIP at two levels: high (H- level; 100 mg/kg CTC + 20 mg/kg SDZ + 20 mg/kg CIP) or low (L- level; 10 mg/kg CTC + 2 mg/kg SDZ + 2 mg/kg CIP). A control treatment was also prepared without the addition of antibiotics. Saw dust (<4 mm) was mixed with swine manure (1: 1 DW) to adjust the C/N ratio to ~29 and moisture content to ~55%; and the mixing with the saw dust reduced the concentration of the antibiotics in the composting mass half of the concentration mentioned above. Therefore the final antibiotic spiked concentrations of the H- level treatment were 50 mg/kg CTC + 10 mg/kg SDZ + 10 mg/kg CIP; and low level (L- level) treatments were 5 mg/kg CTC + 1 mg/kg SDZ + 1 mg/kg CIP). Composting of swine manure was conducted for 56 days with an aeration rate of 0.5 L/kg DW/min. Details of the composting process, organic decomposition, antibiotic degradation and the methods of analyses were presented in a previous report (Selvam et al., 2012).

2.2. DNA isolation and polymerase chain reaction amplification

On predetermined days, the genomic DNA of the composting mass from different treatments were isolated from about 200 mg fresh sample using QIAamp DNA stool Mini kit (Cat.# 51504, QIAGEN) according to the manufacturer's protocol and used as templates for the subsequent PCR. For each sample, DNA extraction was performed with two replicate samples (whole sample that includes the manure and the saw dust) to compensate for the heterogeneity. Quantity of the extracted DNA was determined using Nanodrop ND-100 spectrophotometer. Further, the A_{260}/A_{280} and A_{260}/A_{230} ratios were also checked to assess the contamination of protein and humic acids, respectively. Total bacterial 16S rDNA, tetracycline resistance genes, sulfonamide resistance genes and fluoroquinolone resistance genes were amplified from the total genomic DNA isolated from compost samples by PCR using the primer pairs listed in Table 1. For the RT-PCR analysis, replicate DNA extracts were individually analyzed and the results are mean of two replicate samples. The PCR master mix (Promega) was used for 25-μl reaction mix and the reactions were performed using Thermal Cycler PTC0200G (Biorad) with the following reaction conditions: 95 °C for 5 min, followed by 34 cycles at 95 °C for 30 s, 55 °C for 30 s, 72 °C for 1 min, and a final extension step at 72 °C for 10 min. For the PCR amplification of *gyr*A genes, an annealing temperature of 45 °C with an additional 1.5 mM $MgCl_2$ in the reaction mix was used. The PCR products were analyzed by electrophoresis on 2% (w/v) agarose gel with 100 bp DNA ladder (Promega) to confirm the size and the approximate quantity of the amplicons.

2.3. Plasmid construction

The PCR products of the V3 region of the bacterial 16S rDNA and the individual antibiotic resistant genes were purified by Wizard® SV Gel and PCR Clean-Up System (Cat.# A9281, Promega), and then cloned into pGEM-T vector (Promega). The ligation reactions were performed according to the manufacturer's instructions, and 2 μl of each ligation product was transformed into *E. coli* DH5α competent cells. Plasmid DNA of selected transformants was purified by PureYield Plasmid Miniprep System (Cat. # A1222, Promega), sequenced for the confirmation, concentration of the DNA was measured using Nanodrop spectrophotometer, and used as standards for subsequent real-time (RT)-PCR reactions.

2.4. Real-time PCR

Normal PCR was performed to preliminarily screen the existence of 13 tetracycline genes (RPP class: *tet*M, *tet*O, *tet*Q, *tet*S, *tet*T, *tet*W and *tet*B/P; Efflux class: *tet*C, *tet*E, *tet*G, *tet*H, *tet*Yand *tet*Z, refer Levy et al., 1999 for the details on nomenclature), 6 sulfonamide resistance genes (*sul*1, *sul*2, *sul*3, *dfr*A1, *drf*A2 and *drf*A7) and 2 fluoroquinolone resistance genes (*gyr*A and *par*C) from all the genomic DNA samples isolated from composting mass. The *tet* genes were selected based on the prevalence data of the previous reports (Aminov et al., 2001,2002; Chee-Sanford et al., 2001; Mackie et al., 2006). For the sulfonamides and the fluoroquinolones, the most common genes were included in the screening. Six tetracycline resistance genes (RPP class: *tet*W and *tet*Q; Efflux class: *tet*C, *tet*G, *tet*Y and *tetZ)*, four sulfonamide resistance genes (*sul*1, *sul*2, *drf*A1 and *drf*A7) and two fluoroquinolone resistance genes (*gyr*A and *par*C) were positively detected in the screening (data not shown); therefore they were quantified using RT-PCR. The absolute copy number of each ARG was quantified by referring to the corresponding standard curve obtained by plotting copy number of the constructed ARG-carrying pGEM-T plasmid versus threshold cycles. The concentrations of the tested ARGs were presented as percentage of "copy number of an ARG/copy number of 16S rDNA" for each sample in order to emphasize the relative abundance of the resistance genes. Although the copy number of 16S rDNA per bacterial genome can vary among the bacteria (Case et al., 2007), its quantification has previously been used to estimate the overall bacterial abundance and to normalize resistance genes to the bacterial population in environmental samples (Alexander et al., 2011; Mackie et al., 2006; McKinney et al., 2010).

The plasmid DNA of 16S rDNA as well as ARGs were serially diluted for making standard curve. A master mix for each primer set was prepared such that each well contained the following: