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Community structure and population dynamics of ammonia oxidizers in composting processes of ammonia-rich livestock waste☆

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ABSTRACT

This study investigated the relationship between the population dynamics of ammonia-oxidizing bacteria (AOB) and archaea (AOA), and changes in the concentrations of nitrogenous compounds during ammonia-rich livestock waste-composting processes. The data showed that ammonia in beef and dairy cow livestock waste-composting piles was slowly oxidized to nitrite and nitrate after approximately 21–35 days under thermophilic or moderately thermophilic and mesophilic conditions. Real-time quantitative PCR (qPCR) assays showed a relative abundance of betaproteobacterial AOB during ammonia oxidation but did not detect AOA in any composting stage. Furthermore, real-time qPCR and terminal-restriction fragment length polymorphism (T-RFLP) analyses for the AOB in two composting processes (beef and dairy cow livestock waste) out of the three studied found that thermophilic or moderately thermophilic uncultured betaproteobacterial AOB from the "compost AOB cluster" contributed to ammonia oxidation during hot composting stages. Non-metric multidimensional scaling analyses of the data from T-RFLP showed that only a few analogous species predominated during composting of beef, dairy cow and pig livestock wastes, and thus, the AOB community structures in the three composting piles operating under different conditions were similar. AOB-targeted clone library analyses revealed that uncultured members of the "compost AOB cluster", which could be clearly distinguished from the authentic species of the genus *Nitrosomonas*, were the major constituents of the AOB populations. These results suggested that a limited and unique species of AOB played a role in ammonia oxidation during the composting of ammonia-rich livestock waste.

Introduction

Composting can be used to convert livestock farming waste into nutrient-rich biofertilizers that are effectively accessible to plants. Fresh livestock waste contains a large amount of organic nitrogenous compounds (e.g. proteins, nucleic acid, and amino acids), which are easily mineralized to produce ammonia via hydrolysis of urea, proteins and nucleic acids, and/or deamination of unincorporated nitrogenous organic compounds in the composting process. Ammonia produced in composting piles tends to change the pH to alkaline, and is easily vaporized as free ammonia (NH_3) during the high-temperature stage (>70 °C) and under high pH conditions (Fukumoto et al. 2003; Osada and Fukumoto 2001). Once NH_3 is released into the environment, it can be dissolved in rainwater and can cause eutrophication in closed water areas (Schulze et al. 1989). Several technologies have been used to diminish NH_3 emission during composting processes, including biofiltration (Jun and Wenfeng 2009; Yasuda et al. 2009), the use of chemical scrubbers (Miller and Macauley 1988), and hyperthermophilic pre-treatment (Yamada et al. 2007, 2008).

Residual ammonia in composting piles is utilized in a microbial nitrification–denitrification process, such as assimilation, or for biomass formation in the composting processes. Chemolithotrophic nitrification consists of oxidation of ammonia to nitrite and the subsequent oxidation of nitrite to nitrate, and its efficient progress enhances the production of good compost fertilizer with well-balanced nitrogenous compounds. However, incomplete nitrification leads to an imbalance of nitrogenous compounds in compost fertilizer, and N_2O is generated as a by-product

☆ All the bacterial *amoA* and 16S rRNA gene sequences obtained in this study were deposited in the DDBJ/EMBL/GenBank databases (accession numbers: AB713935 to AB713951). In addition, two archaeal *amoA* gene sequences from the clones used as standards in quantitative real-time PCR assays were deposited in the DDBJ/EMBL/GenBank databases (accession numbers: AB720058 to AB720059).

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when ammonia-oxidizing microorganisms (AOMs) either convert hydroxylamine to nitrite (Otte et al. 1999) or cause denitrification under conditions of low oxygen availability (Arp and Stein 2003; Wrage et al. 2001). The denitrification step caused by denitrifying bacteria results in the crucial emission of N_2O in livestock waste-composting piles (Maeda et al. 2010), although a recent study using isotopomer analysis clearly demonstrated that AOMs also contributed to N_2O emission in the surface layer of composting piles (Maeda et al. 2010). N_2O emissions have, in fact, been observed in many studies of composting processes (Amlinger et al. 2008; Maeda et al. 2010), and this is disconcerting because N_2O is a potent greenhouse gas, which contributes to the breakdown of the ozone layer in the stratosphere (Ravishankara et al. 2009). To date, the amount of livestock waste has increased along with the increase in the human population, and composting of livestock waste is now required in order to produce an important sustainable fertilizer for food production. Thus, biological information about AOMs and nitrite-oxidizing bacteria (NOB) should be accumulated in greater detail for a more comprehensive understanding of biological ammonia oxidation and nitrite oxidation in composting processes using ammonia-rich animal manure.

Biological ammonia oxidation under aerobic conditions has been reported to occur in many natural and engineered ecosystems. The organisms associated with ammonia oxidation are phylogenetically diverse across two domains of prokaryotes. In the domain *Bacteria*, ammonia-oxidizing bacteria (AOB) are associated with the proteobacterial lineage (*Betaproteobacteria* and *Gammaproteobacteria*). In the domain *Archaea*, the phylum *Thaumarchaeota* encompasses ammonia-oxidizing archaea (AOA) (Brochier-Armanet et al. 2008). Some members of the two groups are involved in livestock waste-composting processes (Maeda et al. 2010; Yamamoto et al. 2010, 2011, 2012; Zeng et al. 2011). However, detailed ecological information on the succession of AOMs is still lacking, and little has been described about the common AOM members involved in the composting processes of ammonia-rich livestock wastes. Therefore, the present research focused on the community structure and population dynamics of AOMs during composting of three different types of animal manure by applying molecular and statistical analyses, as well as analysis of physicochemical data obtained during the processes involved.

Materials and methods

Composting setup and compost sampling

Compost samples produced from cow (beef and dairy) dung/sawdust (80%/20% w/v) and pig dung/sawdust (80%/20% w/v) were taken from windrow composting processes in Okinawa, Japan. The three piles (2 m long, 1.8 m wide, 1.2 m high) were prepared on concrete ground under a roof. The piles of beef cow dung/sawdust, dairy cow dung/sawdust, and pig dung/sawdust were operated for 42, 91, or 63 days, respectively, and they were plowed once a week throughout the duration of composting. Compost samples for analysis were collected every 7 days at the center of the horizontal position, at a depth of 20 cm from the top of each composting pile throughout each composting process.

Physicochemical analysis

The compost samples (1 g dry weight) were mixed well with water (10 mL) to obtain supernatants (Nancy 2003), and their pH values were measured. The total volatile solids, moisture content, and dry weight of the compost samples were measured as previously described (Yamada et al. 2007). The C/N ratio of all samples was measured using a CN Corder MT-700 (Yanaco New Science, Kyoto, Japan), as previously reported (Yamada et al. 2007). To measure NH_4^+–N and NO_2^-–N, fresh compost samples (2 g dry weight) and 10 mL of 1 M KCl were mixed by shaking, and NH_4^+–N and NO_2^-–N were measured in the supernatant by using an Ammonium Test kit (MERCK, Darmstadt, Germany) and a Nitrite Test kit (MERCK), respectively, as described previously (Okano et al. 2004; Yamada et al. 2007). To extract nitrate, compost samples (2 g dry weight) were mixed with 10 mL of 0.5 M K_2SO_4. NO_3^-–N in the supernatants was measured using a colorimetric method, as described previously (Anderson and Ingram 1993; Yamada et al. 2007). All measurements were performed in triplicate.

Microorganisms, media, and cultivation

Escherichia coli strain DH5α was purchased from Takara Bio (Takara, Ohtsu, Japan), and *Nitrosomonas europaea* strain ATCC 19718 was obtained from the American Type Culture Collection (Manassas, VA, USA). For the cultivation and isolation of ammonia oxidizers in all composting processes, AOB liquid medium was prepared for *N. europaea* (Krümmel and Harms 1982), which contained NaCl at concentrations of 0, 50, 100, and 200 mM. Agar and gellan gum were used as gelling reagents for the preparation of solid AOB medium (Krümmel and Harms 1982).

DNA extraction

DNA was extracted from the *E. coli* and *N. europaea* strains by using a method previously reported by Hiraishi (1992). DNA from each compost sample was extracted either according to a previous method (Yamada et al. 2005) or using a Power Soil DNA kit (MO BIO, Carlsbad, CA, USA). Sample DNA was also extracted from tea orchard soil (*Camellia sinensis*) and utilized to produce real-time PCR standards (see below). The extracted DNA was purified using a GENECLEAN Turbo kit (Q-Bio Gene, Irvine, CA, USA).

Real-time quantitative PCR

Real-time quantitative PCR (qPCR) assays were carried out using a Light Cycler (Roche, Basal, Switzerland) with SYBR Premix *Ex Taq* Perfect Real Time (Takara). A reaction mixture for the real-time PCR that included 1 ng or 10 ng of template DNA was prepared according to the manufacturer's instructions. The following 16S rRNA gene- and ammonia monooxygenase-alpha subunit (*amoA*) gene-targeted PCR primer sets were used to quantify gene copy numbers by using real-time PCR: EUB338f and 907r (Amann et al. 1990; Lane 1991) for the 16S rRNA gene of domain *Bacteria*, CTO189f and CTO654r (Kowalchuk et al. 1997) for the 16S rRNA gene of betaproteobacterial AOB, amoA1F-amoA2R (Rotthauwe et al. 1997) for the *amoA* gene of AOB, and Arch-amoAF and Arch-amoAR (Francis et al. 2005) and amo111F and amo643R (Treusch et al. 2005) for the *amoA* gene of AOA. The standards to quantify the 16S rRNA gene copies for bacterial prokaryotes and betaproteobacterial AOB were prepared from the PCR amplification products of the 16S rRNA genes of *E. coli* and *N. europaea* with the primer set 27f and 1492r (Weisburg et al. 1991). A standard sample of bacterial *amoA* DNA used in real-time qPCR analysis was produced as described previously (Yamada et al. 2007). The archaeal *amoA* gene standard was prepared by amplifying the archaeal *amoA* gene from the total DNA extracted from tea orchard soil by using Arch-amoAF and Arch-amoAR (Francis et al. 2005) or amo111F and amo643R (Treusch et al. 2005) PCR primer sets. The archaeal *amoA* PCR products were inserted into the pCR4-TOPO vector (Life Technologies, Carlsbad, CA, USA) by using a TOPO TA cloning kit and *E. coli* TOPO10 competent cells (Life Technologies), according to the manufacturer's instructions. The plasmid containing the archaeal *amoA* gene PCR product was linearized with 10 U of *Not* I (Takara) at 37 °C for 3 h prior to use. The DNA