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Minireview

Microbiology of nitrogen cycle in animal manure compost

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Summary

Composting is the major technology in the treatment of animal manure and is a source of nitrous oxide, a greenhouse gas. Although the microbiological processes of both nitrification and denitrification are involved in composting, the key players in these pathways have not been well identified. Recent molecular microbiological methodologies have revealed the presence of dominant *Bacillus* species in the degradation of organic material or betaproteobacterial ammonia-oxidizing bacteria on nitrification on the surface, and have also revealed the mechanism of nitrous oxide emission in this complicated process to some extent. Some bacteria, archaea or fungi still would be considered potential key players, and the contribution of some pathways, such as nitrifier denitrification or heterotrophic nitrification, might be involved in composting. This review article discusses these potential microbial players in nitrification–denitrification within the composting pile and highlights the relevant unknowns through recent activities that focus on the nitrogen cycle within the animal manure composting process.

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Introduction

Composting is the simplest traditional animal manure management technology that depends on the degradation of organic matter by the microbial community within manure itself (Bernal *et al.*, 2009). Easily degradable organic matter would be utilized as the energy source, and CO_2, NH_3 and moisture would be emitted and would generate large amounts of heat; the temperature inside compost piles is about 70°C. The mass of the pile decreases significantly, and the process also reduces odorous compounds and pathogens, while killing weed seeds. Because the mature product can be reused as organic fertilizer, composting is a very important technology from the viewpoint of the circulation of resources or environmental protection.

Because the organic or inorganic state of nitrogen contained within the compost is an important nutrient for crops, the available amount of nitrogen content in composted material is a precious component. Through the composting process, the organic nitrogen contained within initial fresh manure is degraded into ammonium by a wide variety of microorganisms including bacteria and fungi. Part of this nitrogen is lost as NH_3 by volatilization, or through conversion into gases content such as N_2O or N_2 through the nitrification/denitrification process (Fig. 1). The range of nitrogen loss can vary between 19% and 77%, which mainly occur through NH_3 volatilization and N_2 emission (Martins and Dewes, 1992; Mahimairaja *et al.*, 1995; Eghball *et al.*, 1997; Tiquia and Tam, 2000; Tiquia *et al.*, 2002). In addition, 0.2–9.9% of initial nitrogen content can be emitted as N_2O, the intermediate of denitrification or by-products of nitrification (Kuroda *et al.*, 1996; Hao *et al.*, 2001; 2004; Fukumoto *et al.*, 2003; El Kader *et al.*, 2007; Szanto *et al.*, 2007) (Table 1). The loss of nitrogen during animal manure composting processes is affected by various parameters, such as the animal species, diet, bulking agents, moisture content, turning frequency, carbon/nitrogen ratio and initial nitrogen content.

Nitrous oxide is an important greenhouse gas with strong global warming potential (300 times as that of CO_2 (IPCC, 2001). Moreover, because N_2O is greatly responsible for ozone depletion, reduction of its emission would

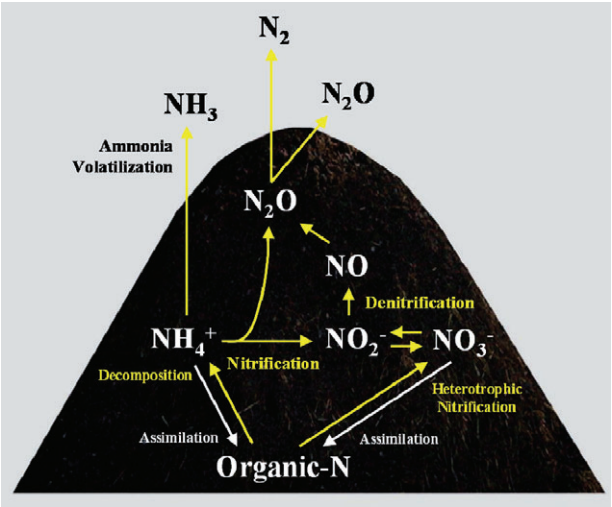

Fig. 1. Nitrogen conversion and emission during the composting process.

be important for environmental protection (Ravishankara *et al*., 2009). Therefore, an important issue in the study of composting is the nitrifier/denitrifier microbial community, which plays a significant role in nitrogen conversion within the composting pile. In this review article, we deal with recent research activities that focus on the nitrifier/denitrifier microbial community in composting while referring to similar studies in other environments.

Overall microbial and fungal community in the composting process

There are many studies about microbial community structures in the composting process. Most of them focus on the bacteria mainly responsible for the degradation of organic matter. In order to identify microbes present in the compost process, besides the classical isolation technique, new approaches based on culture-independent techniques, such as the extraction of DNA from the compost and amplification of 16S rRNA gene by PCR, followed by DNA sequencing are commonly used (Muyzer *et al*., 1993). The approach based on DNA sequencing provides relevant information on microbes that are difficult to culture.

It has been reported that some *Bacillus* species are important in the composting pile in the thermophilic stage, when active degradation of organic compounds occurs (Blanc *et al*., 1997; 1999; Ishii *et al*., 2000; Peters *et al*., 2000; Dees and Ghiorse, 2001; Zhang *et al*., 2002; Ishii and Takii, 2003; Schloss *et al*., 2003; Iida *et al*., 2005; Kim *et al*., 2006; Wang *et al*., 2007; Yamamoto *et al*., 2009). These *Bacillus* can grow and degrade organic compounds under thermophilic conditions up to 65°C, and *Thermus* species are dominant instead of *Bacillus* species above 70°C (Beffa *et al*., 1996). While this is relevant, it should be noted that the bacterial community structure changes dramatically even in the maturing phase, when active degradation of organic compounds has almost ended. In the maturing phase, mesophilic *Proteobacteria* or *Actinobacteria* are known to be dominant (Danon *et al*., 2008), and these bacterial groups are considered responsible for the maturation process.

In the composting process, the temperature in the core zone of the pile reaches 60–70°C, and there are temperature gradient effects within the pile (Fernandes *et al*., 1994). In addition, there is an oxygen gradient and anoxic conditions deep inside the piles (Hao *et al*., 2001), especially in passively aerated composting systems. In these various complicated environments, bacterial communities differ significantly between the surface and the core zone (Maeda *et al*., 2010a). In the dairy cattle manure composting process, nitrite and nitrate accumulate on the surface layer of the pile even in the initial stage of the process, when there are still easily degradable organic compounds (Maeda *et al*., 2010b). In the surface layer, 16S rRNA-dependent bacterial community analysis suggests that some *Proteobacteria* or *Bacteroidetes* are dominant, which is significantly different from the case in

Table 1. Nitrous oxide emission from manure composting process.

Animal	Process type		Unit	Reference
Dairy		0.582	g-N_2O per kg DM	Pattey *et al.* (2005)
Beef		0.162	g-N_2O per kg DM	
Pig	Forced aeration	1.9–71.9	g-N_2O-N per m^3	Osada and Fukumoto (2001)
Cattle	Static	1.1	kg-N per Mg manure	Hao *et al.* (2001)
Cattle	Turned	1.9	kg-N per Mg manure	
Cattle	Woodchip	0.39	%N	Hao *et al.* (2004)
Cattle	Straw	0.68	%N	
Dairy	Static	1.2	%N	El Kader *et al.* (2007)
Dairy	Turned	1.9	%N	
Turkey		0.2–0.4	%N	
Pig	Turned	3.7–4.6	%N	Fukumoto *et al.* (2003)
Pig	turned	2.5	%N	Szanto *et al.* (2007)
Pig	static	9.9	%N	

thermophilic core zones. Some part of these bacterial species are thought to contribute to the nitrification or denitrification that actively occurs in the surface layer.

Although Dees and Ghiorse (2001) reported that they failed to detect archaea in the compost piles, while they found many fungal species in the compost samples whose temperatures did not exceed 50°C. In this regard, Anastasi and colleagues (2005) reported the isolation of 194 fungal species, the *Acremonium*, *Aspergillus*, *Cladosporium*, *Malbranchea*, *Penicillium*, *Pseudallescheria* and *Thermomyces* species from compost. In another study, Hultman and colleagues (2009) reported that fungal biomass can represent between 6.3% and 38.5% of total biomass in municipal waste compost based on phospholipid fatty acid analysis. They also found that the fungal community suffers dramatic changes during the composting process, as does the bacterial community, and that a fungal community succession differed between a full-scale composting facility and a laboratory-scale small reactor. Studies are needed on the function of the fungal community in the degradation of organic matter in the huminification process, or potential interaction with the bacterial community and its contribution to the nitrification/denitrification pathway.

Microorganisms relevant to the nitrogen cycle in composting

Nitrifiers

Nitrification is known to be carried out by bacteria, archaea and fungi (De Boer and Kowalchuk, 2001; Leininger *et al*., 2006; Laughlin *et al*., 2008). In the bacterial process, nitrification consists of two steps, ammonia oxidation and nitrite oxidation, and each of these reactions is performed by an individual microbial group: ammonia-oxidizing bacteria (AOB) (Kowalchuk and Stephen, 2001) and nitrite-oxidizing bacteria (NOB) respectively. Nitrous oxide is known to be produced as a by-product of hydroxylamine oxidation. betaproteobacterial AOB or *Thaumarchaeota* ammonia-oxidizing archaea (AOA) are considered important in ammonia oxidation (Brochier-Armanet *et al*., 2008), and the major NOB in the environment are alphaproteobacterial *Nitrobacter* or *Nitrospira*. Nitrifiers grow slowly under laboratory conditions, and their cultivation or isolation is very time-consuming. In order to speed the tracking of these microbes, a molecular biology approach using primers specific to the 16S rRNA genes and the ammonia monooxygenase gene of betaproteobacterial AOB have been developed and used to track nitrifiers in the environment (Innerebner *et al*., 2006; Junier *et al*., 2010).

In the composting process, temporal nitrite accumulation in the middle stage and high accumulation of nitrate in the mature phase were observed (He *et al*., 2000; 2001; Fukumoto *et al*., 2006; Fukumoto and Inubushi, 2009; Maeda *et al*., 2010c), and it is evident that nitrification occurs in the compost pile. However, it remains unclear which microbes are responsible for this process. Some studies have detected sequences similar to known AOB species *Nitrosomonas europaea-eutropha* or *Nitrosospira* in the composting process (Kowalchuk *et al*., 1999; Jarvis *et al*., 2009; Maeda *et al*., 2010b). Jarvis and colleagues (2009) also detected *Nitrosomonas* in the theromophilic stage and *Nitrosospira* in the maturation phase of household waste composting, while Maeda and colleagues (2010b) detected *Nitrosomonas* throughout the process, especially from the surface layer of a cattle manure composting pile. Some previous studies also detected *Nitrosomonas* from landfill cover, an organic-rich environment similar to the composting process (Mertoglu *et al*., 2006; Zhu *et al*., 2007). Betaproteobacterial AOB are chemoautotrophic and generate energy from the hydroxylamine oxidation step, the ATP produced is used to fix CO_2 as a carbon source. Therefore, the presence of these AOB indicates that these bacteria oxidize ammonia in the composting process. However, the extent of the contribution to net nitrification is yet unknown.

To clarify the contribution of AOB to ammonia oxidation in composting, the contribution of AOA must be studied. Although AOA is known to be responsible to some extent for nitrification in environments containing less organic matter, such as soil, ocean or river sediment (Leininger *et al*., 2006; Santoro *et al*., 2010), there are some reports that AOB's contribution is much more important than AOA's for actual nitrification in organic-rich environments such as wastewater from treatment plants (Park *et al*., 2006; Wells *et al*., 2009). Another report shows that ammonia oxidation in zinc-contaminated soil is restored not by AOA but by AOB (Mertens *et al*., 2009). Many heavy metals are included in livestock manure, especially in swine manure (Nicholson *et al*., 1999; Ko *et al*., 2008). AOB might be more important than AOA under these conditions. Although a previous report failed to detect the AOA in compost piles (Maeda *et al*., 2010b), Yamamoto *et al*. (2010) reported the existence of AOA from cattle manure compost in the later stage of the process. The relative contribution of AOB and AOA on actual nitrification needs to be clarified.

Heterotrophic nitrification is a reaction in which heterotrophic bacteria oxidize ammonia or degrade organic matter to nitrate directly (Papen and Von Berg, 1998). Many bacterial species are known to undergo this reaction, and species such as *Paracoccus denitrificans* or *Pseudomonas putida* are known to possess *amoA* sequences distinct from those of autotrophic nitrifiers (Moir *et al*., 1996; Daum *et al*., 1998). Although these bacteria have potential to contribute net nitrification in the compost, its actual contribution is not known at all. This

reaction by heterotrophs has not been considered in depth because these species do not generate energy from this process, nor do they accumulate high concentrations of nitrite; however, this futile reaction may be of relevance in an environmental setting. These heterotrophic nitrifiers assimilate more ammonium than chemoautotrophic AOB, which leads to higher biomass, and they have been considered not useful for wastewater treatment systems (Podmirseg *et al*., 2010). Efforts to unveil nitrification process include the development of new culture media for thermophilic nitrifiers in compost under heterotrophic conditions is ongoing (Shimaya and Hashimoto, 2008). In summary, it can be said that to understand the nitrogen cycle in the composting process, we need to learn more about the role of the heterotrophic nitrifiers in the process.

On the other hand, there have not been many molecular ecological studies on NOB. Because NOB has a diverse taxonomy, including *Nitrobacter* (α-*Proteobacteria*), *Nitrosococcus mobilis* (γ-*Proteobacteria*), *Nitrospina gracilis* (δ-*Proteobacteria*) and *Nitrospira* (*Nitrospira*), it is difficult to detect all these strains by methods such as FISH (fluorescent *in situ* hybridization) or PCR, which depend on 16S rRNA sequences. Few studies have focused on functioning gene sequences of nitrite oxidoreductase of *Nitrobacter* (Poly *et al*., 2008; Wertz *et al*., 2008). Even though these methods may be effective for understanding nitrification in the composting process, they have not been used for studies of nitrite oxidation yet, and NOB in the composting process has not been characterized well.

Denitrifiers

Nitrite or nitrate generated by nitrifiers would usually be reduced by heterotrophic denitrifiers and emitted into the atmosphere as N_2O or N_2. Denitrification by bacteria has been well studied and the details of its molecular mechanisms have been characterized. The reaction consists of four reduction steps, namely, $NO_3 \rightarrow NO_2 \rightarrow NO \rightarrow N_2O \rightarrow N_2$. The genes *nar*, *nir*, *nor* and *nos* are coding the catalysing enzymes (Rudolf and Kroneck, 2005; Tavares *et al*., 2006). Denitrifying bacteria are known to be phylogenetically diverse, with at least 50 genera (Zumft, 1997). Therefore, the study of denitrifiers that depend on 16S rRNA gene sequences is very difficult, and functioning genes that code each enzyme catalysing each denitrification step are frequently used for studies of environmental denitrifiers (Sharma *et al*., 2005; Wertz *et al*., 2009). Because of the relative abundance of information in public databases, nitrite reductase (*nirS* and *nirK*) or nitrous oxide reductase (*nosZ*) have been used frequently.

There are two types of nitrite reductase: *nirS*, cytochrome c nitrite reductase, which has haem iron in its active centre (Einsle *et al*., 1999; 2000), and *nirK*, a copper-containing nitrite reductase (Murphy *et al*., 1997; Antonyuk *et al*., 2005). It is possible to distinguish these nitrite reductases in denitrifiers by using diethyldithiocarbamate (DDTC), which chelates copper of the *nirK* denitrifier and prevents the process. DGGE primers targeting nitrite reductase gene sequences have been developed (Throback *et al*., 2004) and used to study denitrifiers in various environments. It is frequently discussed which types of nitrite reducers would be dominant in the environment. For example, one report shows that *nirS* denitrifiers are dominant in subtropical macrotidal estuaries (Abell *et al*., 2009). Because the horizonal transfer of denitrifying genes may occur within the environment, the incidence of *nirK* or *nirS* does not always agrees with the 16S rRNA gene phylogenetic sequences. Heylen and colleagues (2006a) concluded that *nir* genes may not be suitable to evaluate microbial diversity of denitrifiers in the environment. Thus, interpretation of biodiversity based on *nir* sequence analysis need to be interpreted with care.

Nitric oxide reductase (NOR) catalyses the reduction of NO to N_2O. Nitric oxide, produced by the reduction of nitrite, is known to be toxic to microorganisms, and they need to metabolize it to protect themselves. Three kinds of NOR have been reported, namely cNOR, qNOR and qCuNOR (Tavares *et al*., 2006). The cytochrome c-dependent nitric oxide reductase (cNOR) of *P. denitrificans* has been studied well. It is a component of cytochrome bc complex with two non-haem irons in its active centre. Braker and Tiedje (2003) were the first to study denitrifying communities using *norB* as a functional marker, and others have used it for studies on environmental samples (Dandie *et al*., 2007) or isolates (Heylen *et al*., 2006b), but not yet for compost samples.

Nitrous oxide reductase is the terminal oxidoreductase of denitrification that transforms N_2O to N_2 (Brown *et al*., 2000). Because this multi-copper-containing enzyme prevents the accumulation of a potent greenhouse gas, it plays an important role in the nitrogen cycle (Zumft, 2005). Nitrous oxide reductase is most sensitive to molecular oxygen among the enzymes involved in denitrification, and its function is inhibited under aerobic conditions. This enzyme can also be inhibited by C_2H_2 easily (Yoshinari and Knowles, 1976) and is frequently used for the study of the denitrification potential of environmental samples (Teissier and Torre, 2002). Moreover, the *nosZ* gene that codes this enzyme is used as a biomarker in molecular ecological studies (Scala and Kerkhof, 1999; Stres *et al*., 2004).

Some bacterial denitrifiers and fungi are known not to possess nitrous oxide reductase (Takaya, 2009). Although N_2O reduction is thermodynamically favourable and N_2O is suitable for an electron acceptor, some denitrifiers produce N_2O as the final product of the denitrification process. This might be explained by the fact that nitrous

oxide is not toxic to microorganisms, whereas NO is toxic to bacterial cells. The lack of N_2O reduction makes ~20% difference to the bioenergetics of the bacterium (Richardson *et al*., 2009).

Diversity of denitrifiers in the environments

A study about denitrifier communities in the composting process revealed an initial variation of *nirK* diversity and stability after that (Maeda *et al*., 2010a). Hallin and colleagues (2006) also reported that the addition of methanol or ethanol to activated sludge significantly affected the diversity of *nirS* but not that of *nirK*. On the other hand, the addition of mature compost that contains NO_2 or NO_3-N did not affect *nirK* diversity but significantly affected *nosZ* diversity, suggesting that denitrifiers possessing the *nosZ* gene in the compost would be more sensitive to environmental conditions. It is necessary to isolate the major denitrifier revealed by molecular methods in order to understand the actual denitrification occurring in the environment. In a denitrifier community study of rice paddy soil, Ashida and colleagues (2010) successfully isolated a major denitrifier through the enhancement of denitrification activity with succinate amendment and molecular methods such as the 16S rRNA gene clone library approach (Ishii *et al*., 2009) or the stable isotope probing approach (Saito *et al*., 2008). Moreover, Ishii and colleagues (2011) proved that denitrifiers with different 16S rRNA gene phylogeny possess same *nirS* or *nirK* gene in the same environment (Fig. 2). Their data show previously unknown complex relationship between 16S rRNA gene and functional gene possession. To understand the denitrifier community completely, it is necessary to combine independent approaches such as molecular and conventional cultivation approaches. The molecular methods used to characterize the unknown and uncultivated denitrifier communities, and the subsequent single-cell isolation strategy would be effective for the denitrifiers that are truly functioning for actual denitrification in the environment (Ishii *et al*., 2010).

The relationship between denitrifying gene diversity or abundance and potential denitrification activity in soil has been well studied. Potential denitrification activity, $N_2O/(N_2O + N_2)$ and the denitrifier community would be affected by pH (Palmer *et al*., 2010). Cuhel and colleagues (2010) reported that *nirS* diversity correlates with soil pH. On the other hand, Hallin and colleagues (2009) reported that denitrification activity did not correlate with denitrifier gene composition, but did correlate with the size of the total bacterial community or *nosZ* abundance. Another study reports that the *nosZ* ratio to total bacterial community is much more important than denitrifying gene abundance for potential N_2O production (Philippot *et al*., 2009). Although much effort has been made in these environmental studies, it is still difficult to explain denitrification and nitrous oxide production by denitrifying gene abundance or diversity. Moreover, a previous paper reported that AOA possesses a novel *nirK* sequence (Bartossek *et al*., 2010), which had not been covered by previous denitrifier studies, and much effort should be made to learn about this unknown archaean denitrification.

It is also known that some autotrophic nitrifiers have the ability to denitrify (Wrage *et al*., 2001; Wrage *et al*., 2004; Shaw *et al*., 2006). These autotrophic nitrifiers possess *nirK*-type nitrite reductase with distinct DNA sequences from those of heterotrophic denitrifiers. It is not well understood yet how these autotrophic nitrifiers acquired the *nirK* gene, which might have occurred by horizontal gene transfer; and how they became tolerant to nitrite produced by themselves (Casciotti and Ward, 2001). Because nitrifiers produce nitrite, they have many advantages for the utilization of nitrite as the substrate for denitrification. Therefore, nitrifier denitrification may contribute much more than heterotrophic denitrifiers, but it is difficult to distinguish these pathways with the current analytic techniques.

Who is responsible for nitrous oxide emission?

It is hard to understand how to share NO in nitrification and denitrification procedures. As stated above, nitrification would be performed by AOB, AOA, heterotrophic nitrifiers or fungi, whereas denitrification would be performed by heterotrophic denitrifiers, denitrifying fungi and autotrophic/heterotrophic nitrifiers. However, the relative contribution to net nitrification or denitrification of each group is not yet clear. Studies that focus on nitrifying genes are only about AOB or AOA, and those for denitrifying gene analysis are only about bacteria. The development of a tool for such study will be needed.

Stable isotope analysis of N_2O is an alternative approach to studying its production processes because the relative abundance of stable isotopes is a function of their abundance in source materials and the isotope fractionation factor of each physical/chemical process. In particular, intramolecular ^{15}N distribution within the N_2O molecule (site preference, SP) has been found to depend only on enzymatic reaction processes and not on substrates (Toyoda and Yoshida, 1999; Yoshida and Toyoda, 2000; Toyoda *et al*., 2005; Sutka *et al*., 2006). Nitrous oxide, which originates from bacterial nitrification (hydroxylamine oxidation) and denitrification (nitrite reduction), can be distinguished by using SP. However, SP cannot distinguish between nitrifier denitrification and heterotrophic denitrification, and a recent study showed that fungal denitrification produces N_2O with SP similar to that of bacterial nitrification (Sutka *et al*., 2008). In addition, isotope abundance is affected by nitrous oxide

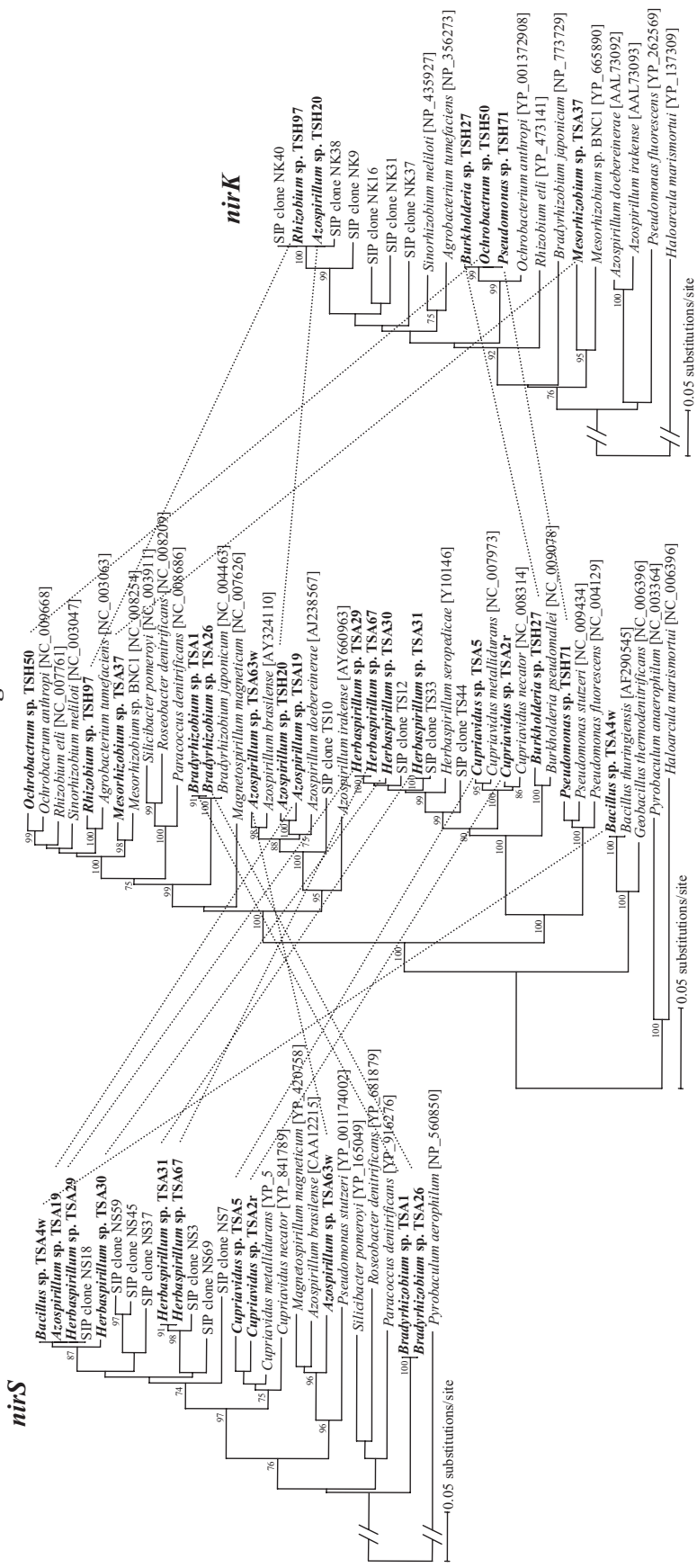

Fig. 2. The relationship of 16S rRNA gene and functional denitrifier genes (*nirS*, *nirK*) possession of denitrifiers isolated from rice paddy soil (Redrawn from Ishii *et al.*, 2011). Isolated strains were written in bold, and the possession relationship was connected by dotted lines.

reduction (Ostrom *et al*., 2007; Jinuntuya-Nortman *et al*., 2008). Although this analytical technique has some limitations as stated above, it would be a powerful tool by using all available isotopic data (N and O isotope ratios and SP) in a complementary style (e.g. Koba *et al*., 2009) or by combining other analytical approaches, such as a wide range of molecular methods. Isotopomer analysis of N_2O directly collected from a composting pile by the dynamic chamber method (Osada and Fukumoto, 2001) revealed that bacterial denitrification is the most important and responsible nitrous oxide production pathway (Maeda *et al*., 2010b). This study relied on the isotopic characteristics of N_2O produced by isolates not from compost but from other environments (such as soil). Therefore, in future studies, we need to isolate the major nitrifier, denitrifier, denitrifying fungi and isotope signature of their producing N_2O.

Future perspective

Because of the ease with which it is managed, composting will continue to be a major technology for treating animal manure. However, analysing the techniques developed previously cannot explain the nitrogen cycle and nitrous oxide emission yet. In the future, combining distinct approaches such as molecular methods, stable isotope analysis and classical isolation techniques will help us to understand the nitrogen cycle during the composting process in detail. The results should lead to the development of relevant mitigation strategies, which will include: identification of the main players in the nitrification–denitrification process in the composting piles; isolation of these key players; and analysis of their physiological, biochemical and ecological properties. It would be also of interest to identify the nitrous oxide reducers and to study their function in the composting piles.

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