論文の内容の要旨

応用生命化学専攻
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論文題目

Identification of N_2O-generating microorganisms in upland field after nitrogen fertilization

（窒素施肥畑圃場における一酸化二窒素ガス生成微生物の特定）

Chapter 1. General introduction

Nitrous oxide (N_2O) is known as a powerful greenhouse gas (GHG), which generates a 298-fold stronger effect on global warming than carbon dioxide (CO_2). N_2O can be photolyzed into nitric oxide (NO) in the stratosphere, which contributes to acid rain and involve in stratospheric ozone depletion. Therefore, N_2O has long drawn substantial attention in field of environmental science. Soil accounts for 62% of global N_2O emission, and upland soils applied with N fertilizers contribute mainly to the total soil N_2O emissions because of the large enhancement of microbial N_2O-generating activities by N input. Therefore, we suggest that upland field soil applied with different types and management practices of N fertilizers acts as a mainly N_2O source.

N_2O is known to be produced by soil microorganisms via nitrification and denitrification pathway. To clarify the emission rate and pathway of N_2O in upland field, many strategies and methods were established and developed. However, some limitations of these methods lead us underestimate and misunderstand the regularity of N_2O emission and related controlling factors in upland field soils. For example, the current *nirK* and *nirS* primers, widely used for detecting the nitrite reductase gene associated with N_2O emission, amplified a limited range of bacterial denitrifiers and mismatch not only substantial bacterial denitrifiers but also archaeal and fungal denitrifiers; isotopomer analysis cannot clearly distinguish the contribution between the nitrification and fungal denitrification. Thus, to obtain a comprehensive and precise understanding of the regularity of N_2O emission in upland field soil, we should improve the methodology and combine several research strategies into a multiple analysis for clarifying the emission rate and pathway of N_2O in upland field soil.

Thus, the objective of this thesis was to assess the N_2O emission rate and pathway, and then identify N_2O-generating microorganisms in upland field soil after the basal and additional application with organic or chemical fertilizers, through the multiple analysis methods including the observation of environmental factors, isotopomer ratio, SIR inhibition and acetylene inhibition analysis of N_2O, and abundance and

expression of soil microbial genes associated with N_2O emission. The knowledge and methodology obtained and developed in this thesis will lead us to a more comprehensive understanding of microbial communities involved in N_2O generation and consumption in upland field soils.

Chapter 2. Greater diversity and abundance of prokaryotic denitrifiers in upland field soil than previously realized

In the denitrification process, nitrite reduction to nitric oxide (NO) is a crucial step catalyzed by nitrite reductases (NirK and NirS). The NirK and NirS genes (*nirK* and *nirS*) have been used as marker genes to study the distribution, abundance, diversity and activity of denitrifiers in the environment. However, our phylogenetic analysis of the currently available full-length sequences of prokaryotic *nirK* and *nirS* revealed that conventional PCR primers can detect only a limited variety of the genes. We therefore designed new primer sets that cover the full diversity of prokaryotic *nirK* (Cluster I to IV) and *nirS* (Cluster I to III), including sequences that have been unaccounted for to date. DNA-based clone library and quantitative PCR analyses that used the newly designed primers revealed that prokaryotic *nirK* and *nirS* sequences distributed in terrestrial environments are more phylogenetically diverse and 2-6 times more abundant than previously counted. An RNA-based study that used the newly designed primers combined with culture-based method suggested that prokaryotes carrying the previously unaccounted for *nirK* or *nirS* play an important functional role in denitrification, especially N_2O emitters, in cropland soil. These results indicate that we have underestimated the role of prokaryotic denitrifiers in the environment. The knowledge and methodology obtained and developed in this chapter will lead us to a more comprehensive understanding of the ecology of prokaryotic denitrifiers in environments.

Chapter 3. Unaccounted diversity and abundance of fungal denitrifiers in upland field soil

Fungal denitrification in soils is receiving considerable attention as one of the dominant N_2O production processes, because N_2O, not N_2, is the end product of fungal denitrification. However, because of the lack of a methodology to detect fungal denitrification-related genes, the diversity and ecological behavior of denitrifying fungi in soil remains unknown. Thus, we here designed a primer set to detect the fungal nitrite reductase gene (*nirK*) based on the homologs of the copper center type 1 domain used for the primer design of prokaryotic *nirK* in Chapter 2, which allow us compare fungal *nirK* sequences with the massive store of bacterial *nirK* sequences. We validated the sensitivity and specificity of primers by using fungal and bacterial and archaeal strains having the N_2O producing activity. Then, through clone library analyses, we identified congruence between phylogenies of the fungal 18S rRNA gene and *nirK* of denitrifying fungal isolates, and affirmed the *nirK* of the most dominant denitrifying fungal group in soil (Ascomycota) can be sufficiently detected. The methodology developed here allows to precisely identify denitrifying fungi and to elucidate the importance of fungal N_2O emission in upland field.

Chapter 4. N_2O emission and related controlling factors in upland field soil after N fertilization

Upland field soils mainly contribute to the total N_2O emissions from soil environments, because

substantial microbial N_2O emissions are greatly stimulated by basal and additional organic or chemical N fertilization. In this chapter, based on an observation in upland field soil with corn cultivation in 2011, we found substantial N_2O emission was induced by both basal and additional N fertilization. After basal organic or chemical fertilization, N_2O was produced mainly by denitrification more than nitrification. In such denitrification, the prokaryotic and fungal denitrifiers having *nirK* (Cluster I and II) were mainly responsible for N_2O emission induced by basal organic fertilization, and prokaryotic and fungal denitrifiers respectively having *nirS* (Cluster II) and *nirK* were mainly responsible for N_2O emission induced by basal chemical fertilization. In such nitrification, the nitrifiers having the archaeal *amoA* as the miner N_2O emitters were responsible for organic or chemical fertilizers. In addition, denitrifiers having the *nosZ* in Cluster I as the N_2O reducers in denitrification induced by only organic fertilizers. In contrast, after surface additional organic or chemical fertilization, N_2O was produced dominantly by denitrification and fungal denitrifiers play a dominant active role in N_2O emission and prokaryotes were inactive as the N_2O emitters and reducers because of the O_2 availability.

Chapter 5. Temporal variation of microorganisms and their pathways responsible for N_2O emission in upland field soil after basal N fertilization

As described in chapter 4, N_2O emissions induced by basal N fertilization are always concentrated in several weeks after fertilization, and such concentrated N_2O emission performs significantly larger contribution to total N_2O emission than that by additional fertilization. We performed a field experiment in upland field in 2012 with an exaggerated application of N fertilizer and the prolonged field-scale and lab-scale observation and investigation, which allow us to determine the diverse microorganisms and microbial pathways responsible for N_2O emission induced by basal N fertilization and the temporal variation of such N_2O emission were induced by different environmental factors as follow, (i) after the basal organic fertilization, firstly emitted N_2O was produced mainly via denitrification more than nitrification. Bacterial denitrification, performed by denitrifiers having the prokaryotic *nirK* in Cluster I, II, III and IV and *nirS* in Cluster II, contributed more to N_2O emission than fungal denitrification. The minor N_2O emission via nitrification was mainly produced by AOB; (ii) after the firstly emitted N_2O induced by basal organic fertilization, N_2O was still produced mainly via denitrification more than nitrification, but fungal denitrifiers contributed more to N_2O emission than bacterial denitrification; (iii) after the basal chemical fertilization, firstly emitted N_2O was produced slightly and induced mainly via denitrification more than nitrification. Bacterial denitrification, performed by denitrifiers having the prokaryotic *nirK* in Cluster I, II and III and *nirS* in Cluster II, contributed more to N_2O emission than fungal denitrification. The minor N_2O emission via nitrification was mainly produced by AOB; (iv) after the firstly emitted N_2O induced by basal chemical fertilization, N_2O was produced largely mainly via denitrification more than nitrification following a rainstorm, but fungal denitrifiers and bacterial denitrifiers having *nirS* in Cluster II and bacterial nitrifiers having *amoA* contributed equally to N_2O emission; (v) the denitrifiers having *nosZ* in Cluster I as the N_2O reducers play a crucial role in final amount of N_2O emission. The high expression of such denitrifiers only occurred during the first peak period of N_2O emission after the basal organic fertilization because of the

sufficient organic carbon and low soil pH level, which lead to an equally released amount of N_2O during the first and second peak period of N_2O emission.

Chapter 6. N_2O emission from upland field soil through fungal denitrification after additional organic fertilization

This chapter focused on the large N_2O emission from upland field soil that occurs after surface additional organic fertilization. N_2O emissions following surface organic fertilization were suppressed by 84 and 20% after the addition of cycloheximide (a fungal inhibitor) and streptomycin (a bacterial inhibitor), respectively, suggesting that fungi provide the main contribution to the observed N_2O emission. Thirty-four fungal strains were isolated from the soils, and their N_2O producing activities were analyzed. The abundance of fungal population and fungal denitrifiers in the surface-fertilized soil was much higher than that in the non-fertilized soil. In addition, the fungal community compositions of the soils differed. *Actinomucor elegans*, *Bionectria ochroleuca*, *Fusarium avenaceum*, *Fusarium equiseti*, *Fusarium oxysporum*, *Fusarium solani* and *Nectria* sp. dominated the surface-fertilized soil, and their activity in producing N_2O was confirmed. In addition, based on functional gene markers, fungi belonging to Eurotiales, Hypocreales, and Sordariales were primarily responsible for N_2O emissions in soils. These results suggested that N_2O emission after the surface application of granular organic fertilizers in the cropland field mainly resulted from fungal denitrification.

Conclusion

In the tested upland field soil, substantial N_2O emission was induced by application with N fertilization, and microorganisms and their pathway responsible for such N_2O emission were different depending on the types and management practices of fertilizers. During the period of N_2O emission after the basal N fertilization, diverse microorganisms and microbial pathways responsible for such N_2O emission and temporal variation of these microorganisms were induced by different environmental factors. Diverse bacterial denitrifiers in denitrification were dominantly responsible for early N_2O emission, and fungal and bacterial denitrifiers in denitrification and bacterial nitrifiers (in uncultivated soil) or archaeal nitrifiers (in cultivated soil) in nitrification were mainly responsible for latter N_2O emission, because the potential control of soil organic C supply and soil pH level. During the period of N_2O emission after the additional N fertilization, fungal denitrifiers were dominantly responsible for N_2O emission, because the potential control of soil O_2 supply.

Publications

1) Wei, W., Isobe, K., Shiratori, Y., Nishizawa, T., Ohte, N., Otsuka, S., Senoo, K. (2014) N_2O emission from cropland field soil through fungal denitrification after surface applications of organic fertilizer. *Soil Biology and Biochemistry*, 69, 157-167.