

論文の内容の要旨

応用生命化学専攻

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論文題目

Isolation and characterization of iron-reducing bacteria, potential drivers of reductive nitrogen transformation in paddy soils

(水田土壌の還元的窒素変換を担う鉄還元細菌の分離と性状解析)

Chapter 1: Introduction

Paddy soil is the largest anthropogenic wetlands on the Earth and represents a unique ecosystem that cycles between waterlogged and drained states during long-term paddy cultivation. These seasonal cycles between waterlogged and drained conditions of paddy soils further revolve the aerobic and anaerobic conditions, which greatly affects the soil properties and sets in motion a series of unique physical, chemical, and biological processes not found in dryland soils. Reductive nitrogen transformation (RNT) in paddy soils, including denitrification ($\text{NO}_3^- \rightarrow \text{NO}_2^- \rightarrow \text{NO} \rightarrow \text{N}_2\text{O} \rightarrow \text{N}_2$), dissimilatory nitrate reduction to ammonium (DNRA; $\text{NO}_3^- \rightarrow \text{NO}_2^- \rightarrow \text{NH}_4^+$), and nitrogen fixation ($\text{N}_2 \rightarrow \text{NH}_4^+$), is a microorganism-driven process that is crucial for sustainable rice production and environmental conservation, due to the contributions to reduce leaching of nitrogen pollutants (NO_3^- , NO_2^- , and N_2O) into the environment and increase retention of nitrogen-based nutrients (NH_4^+) for rice plants under waterlogged conditions. Thus, the identification of microbial drivers of RNT in paddy soils is pivotal for successful rice production with minimal environmental nitrogen burden.

To investigate RNT microbes in paddy soils, a metatranscriptomic study, with soil RNA extracted from paddy soils in Japan under waterlogged and drained conditions, was previously performed in our laboratory. The results showed that most of the gene transcripts involved in RNT are derived from the order *Deltaproteobacteria*, specifically the genera *Geobacter* and *Anaeromyxobacter*, both known as iron-reducing bacteria. Moreover, other reports also revealed that the *Geobacter* and *Anaeromyxobacter* strains were possible drivers coupling metal reduction with nitrogen cycles based on the culture-independent analysis in paddy soils. However, to date, no such bacteria with a validly published name have been isolated from paddy soils, and their ability to transform nitrogen also has yet to be confirmed. Thus, I performed this study aiming to isolate paddy soil-derived *Geobacter*-like and *Anaeromyxobacter*-like strains and explore their special roles in biogeochemical cycles, especially the functions related to nitrogen transformation.

Chapter 2: Isolation and identification of *Geobacter*-like and *Anaeromyxobacter*-like strains and reclassification of the family *Geobacteraceae*

To obtain isolated iron-reducing bacteria from the paddy soil for the following studies, hundreds of paddy soil or related sediment samples were collected from all over Japan. After multiple enrichment processes under anaerobic conditions, a total of 63 different *Geobacter*-like strains and 3 different *Anaeromyxobacter*-like strains were successfully isolated. Through a polyphasic taxonomy analysis, the 14 of the 63 *Geobacter*-like isolates were identified as novel species and belong to 2 novel genera in the family *Geobacteraceae*, for which we proposed the names as *Geomonas* gen. nov., including 11 novel species and two reclassified species, *Geobacter bremensis* and *Geobacter bemidjiensis*, and *Oryzomonas* gen. nov., including 3 novel species, while the 3 *Anaeromyxobacter*-like strains were characterized as novel species. With further genomic analysis, it was found that the phylogenetic relationships among the species in the family *Geobacteraceae* are paraphyletic and need a taxonomic reclassification. Based on the phylogenetic analysis and genomic identities, the family *Geobacteraceae* was divided into 11 clades, thus, 11 genera containing all of the species in the family *Geobacteraceae* were proposed.

Chapter 3: Habitat preferences and biogeochemical roles of *Geomonas* and *Oryzomonas* strains

Although the members of the family *Geobacteraceae* reported previously were mostly isolated from sediments, especially contaminated terrestrial environments, the two novel genera *Geomonas* and *Oryzomonas* were proved to prefer paddy soils as habitats with high preference scores. Moreover, a meta-analysis based on the public metagenomic data derived from the paddy soils in Japan revealed the genus *Geomonas* rather than the traditional genus, *Geobacter*, is the predominant group related to the nitrogen cycle in the paddy soil, suggesting the great potential of *Geomonas* strains in biogeochemical cycles. Using culture experiments with *Geomonas* and *Oryzomonas* strains isolated in this study, it was confirmed that all tested strains can reduce ferric irons (10 mM) to ferrous irons within 10 days. X-Ray diffraction (XRD) results also showed that these strains transformed ferrihydrite to vivianite, a common mineral in many geological environments. Nitrogen-fixing capacity was tested for these isolated strains using acetylene reduction assay (ARA). ARA values of all tested *Geomonas* and *Oryzomonas* strains were detectable with a range from 0.08 to 4.1 nmol/h/10⁹ cells. The relative high concentration of Fe(III)-NTA was found to strengthen bacterial nitrogen fixing ability, while nitrate and nitrite inhibit the activity completely. Moreover, the isolated *Geomonas* and *Oryzomonas* strains were also found to utilize not only acetate, but also high-molecular cellulose and xylan, the major components of rice straw, as the carbon source to fix nitrogen, indicating that these strains can obtain carbon source from rice straw directly and further couple carbon and nitrogen cycles in paddy soils.

Chapter 4: Confirmation of DNRA pathway and clarification of DNRA-derived N₂O production in *Geomonas* strains

Besides the special functions related to biogeochemical cycles of all studied strains, all of them were also confirmed to contain the complete DNRA pathway, reducing NO₃⁻ to NH₄⁺ with the intermediate NO₂⁻, as well as a small amount of N₂O produced. N₂O is a damaging greenhouse gas that is mainly produced from the nitrogen cycles in agricultural soils. To date, few reports have clearly answered how the N₂O is produced through the DNRA process.

Based on several physiological tests on two *Geomonas* strains, Red111 and Red32, it was proved that N₂O emission is a biotic process and mainly produced from DNRA process via the intermediate, nitrite (ca. 90%), while less from the other intermediate, NO (ca. 10%). These facts suggested that the DNRA process is also an N₂O source in soil environments. However, quantitative analysis revealed that ammonium was the major product, accounting for more than 50% of added nitrate, while N₂O was the byproduct, only accounting for less than 2% of added nitrate, which implied that DNRA is indeed a process to produce ammoniacal nitrogen and maintain the soil fertility. Furthermore, the site preference (SP) of N₂O from the DNRA process was first determined with a value of 46 – 47‰, the highest value reported so far for biologically produced N₂O, indicating the SP value would be a useful tool to identify the N₂O of the DNRA process. Using the transcriptomic analysis along with RT-qPCR, nitrate reductase (Nar) gene cluster was highly expressed under the nitrite treatment, which is consistent with the phenotypic results of N₂O generation. Along with previous reports that *narG* mutant strains produced less N₂O than wild strains, it was supposed that *nar* gene cluster is mostly the genetic driver reducing nitrite to N₂O in *Geomonas* strains (Fig. 1). Besides, the pH and C/N ratio were found to obviously affect the N₂O emission during the DNRA process via the accumulation of NO₂⁻, which might be a useful reference for N₂O emission mitigation in agricultural management.

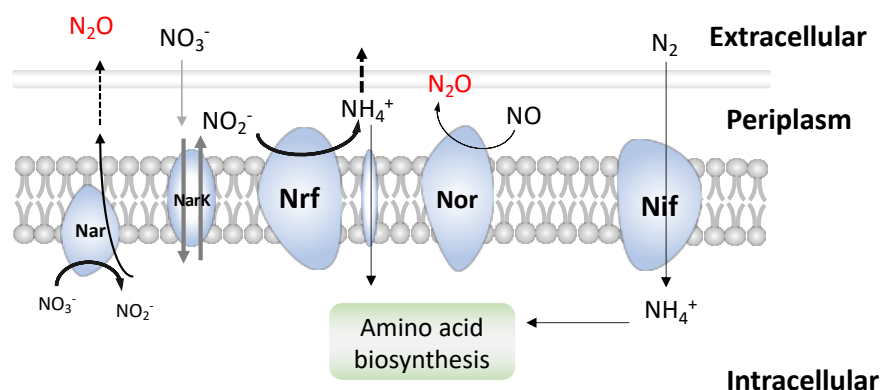
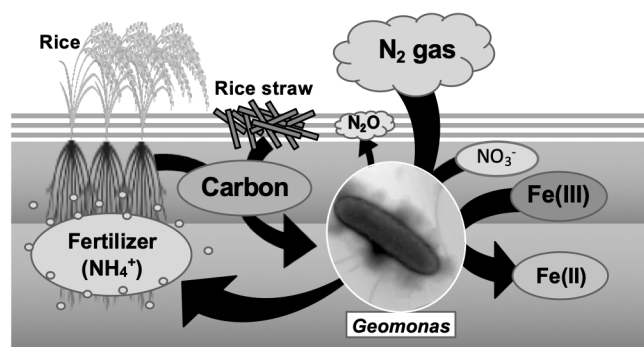


Fig. 1 Schematic description of the N₂O generation from DNRA process

Chapter 5: Conclusion and Prospect

In this study, a total of 63 *Geobacteraceae* and 3 *Anaeromyxobacter* strains from Japanese paddy fields and nearby places were successfully isolated, including 17 novel species and 2 novel genera (*Geomonas* and *Oryzomonas*). At the same time, the phylogenetic positions of all species in the family *Geobacteraceae* were reclassified and all strains were reclustered into 11 genera. Next, the abilities of ferric reduction, nitrogen fixation, cellulose- and xylan-degradation, and nitrate reduction (DNRA) were confirmed with biochemical methods, indicating that these *Geomonas* and *Oryzomonas* strains could be important drivers of the biogeochemical cycle in paddy soil, especially the ammonium production. Finally, based on biochemical and transcriptomic studies, I concluded that N₂O, as a byproduct, is mainly produced from nitrite in the DNRA process, and *nar* gene cluster should be the key role driving N₂O emission, which might be a useful reference for mitigation of N₂O emission in agricultural management. This

study confirmed that iron-reducing microbes are indeed key drivers in nitrogen cycles, especially the ammonium production, implying the great potential of iron fertilization in paddy soils, which would be a reference for sustainable agricultural style of paddy soils with less nitrogenous fertilizer, but higher rice production. However, my studies focusing on function clarification of *Geomonas* strains mainly performed using culture experiments with less consideration of natural conditions they were inhabited, mostly causing some biased results for bacterial description. Therefore, bacterial ecological study *in situ* may be a complementary choice for the following studies.



The metabolic pathway of *Geomonas* in paddy soil

The iron-reducing and nitrogen-fixing bacteria, *Geomonas* spp., absorb carbon source from rice root secretion and rice straw decomposition product and transform N_2 or NO_3^- to rice fertilizer NH_4^+ , maintaining the soil fertility.

Publication:

- [1] **Z. Xu**, Y. Masuda, H. Itoh, N. Ushijima, Y. Shiratori, K. Senoo. *Geomonas oryzae* gen. nov., sp. nov., *Geomonas edaphica* sp. nov., *Geomonas ferrireducens* sp. nov., *Geomonas terrae* sp. nov., four ferric-reducing bacteria isolated from paddy soil, and reclassification of three species of the genus *Geobacter* as members of the genus *Geomonas* gen. nov. *Front. Microbiol.* 2019;10: 2201.
- [2] **Z. Xu**, Y. Masuda, C. Hayakawa, N. Ushijima, K. Kawano. Y. Shiratori, K. Senoo, H. Itoh. Description of *Oryzomonas japonicum* gen. nov., sp. nov., *Oryzomonas niigatensis* sp. nov., and *Oryzomonas ruber* sp. nov., three novel members of the family *Geobacteraceae* isolated from paddy soil and pond sediment. *Microorganisms*, 2020; 8, 634.
- [3] H. Itoh[#], **Z. Xu**[#], Y. Masuda, N. Ushijima, C. Hayakawa, Y. Shiratori, K. Senoo. *Geomonas silvestris* sp. nov., *Geomonas paludis* sp. nov. and *Geomonas limicola* sp. nov., isolated from terrestrial environments, and emended description of the genus *Geomonas*. *Int. J. Syst. Evol. Microbiol.* 2020. Doi: 10.1099/ijsem.0.004607 ([#]Co-first author)
- [4] Y. Masuda, H. Yamanaka, **Z. Xu**, Y. Shiratori, T. Aono, S. Amachi, K. Senoo, H. Itoh. Diazotrophic ability of *Anaeromyxobacter* isolated from soils. *Appl. Environ. Microbiol.*, 86, e00956-20.