Insights into the mechanisms of the differential abilities of two N₂O reducers, *Pseudomonas* sp., isolated from paddy soil

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1 **Insights into the mechanisms of the differential abilities of two N₂O reducers, *Pseudomonas* sp.,**
2 **isolated from paddy soil**

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Abstract

Microbial reduction of nitrous oxide (N₂O) in soil plays an important role in mitigating N₂O emission, and it is the only known biological process for N₂O sink. However, it is not clear about the mechanisms of differential N₂O reducing function of N₂O reducers. In this study, the N₂O reducing activities and *nosZ* gene transcript abundance of two N₂O reducers named *P. veronii* DM15 (DM15) and *P. frederiksbergensis* DM22 (DM22) were determined under varied temperature and oxygen concentration conditions, as well as the whole genomes were sequenced by Illumina sequencing. The results showed that DM15 generally exhibited significantly higher abilities in N₂O reduction than DM22 in regardless of low or high temperature and aerobic or anaerobic conditions. Coincidentally, DM15 expressed significantly more *nosZ* gene transcripts under above environments. Genomic analysis further revealed that DM15 possessed about 30% more transcription related genes than DM22 and the *nos* cluster of the former contained a transcriptional regulator gene of *dnr* which not found in that of the later. Additionally, the *nos* genes of DM15 possessed obviously higher expression potentials (CAI value). In conclusion, the transcriptional regulation of *nos* gene region would be a crucial factor in determining the differences of N₂O reducing abilities of the two N₂O reducing isolates.

Keywords: *Pseudomonas* sp., N₂O reducing ability, *nosZ* expression, genomic analysis, paddy soil

1.Introduction

Nitrous oxide (N₂O) is a major greenhouse gas with high warming potential [1] and posing a threat to the global changes [2]. N₂O consumption is an important process in alleviating N₂O emission [3, 4]. It was discovered that the only known biological N₂O sink is denitrification, in which N₂O is reduced to N₂ by nitrous oxide reductase encoded by *nosZ* gene [5]. In recent years, the community compositions and population size of *nosZ*-containing bacteria in various ecosystems have been investigated, such as grassland [6], forest [7], farmland [8] and sediment [9]. It was demonstrated that the composition and distribution of *nosZ*-containing communities were largely determined by soil pH and other soil properties [10-13], and *nosZ* abundance was positively related to N₂O reduction [14]. Such these discoveries are important in understanding the N₂O consumption and the influenced factors of *nosZ*-containing communities. Since *nosZ* community is composed by diverse bacterial species [15], investigation on the mechanisms of N₂O reduction of *nosZ*-containing strains would be fundamental for the cognition of N₂O reducing functions in ecosystems.

The abilities of N₂O reduction of bacterial strains were influenced by environmental factors including oxygen, temperature, pH and so on [16, 17]. Oxygen was an important factor [18], generally, anaerobic condition was suitable for the functional performance of N₂O reducers, high oxygen concentration could significantly limit the N₂O reductase activity [19]. However, it was also indicated that some aerobic denitrifiers exhibited relatively strong N₂O reducing efficiency under oxic condition [20, 21], such as *Pseudomonas*, *Azospirillum*, *Herbaspirillum*, *Marinobacter* [22-26]. The suitable pH and temperature for N₂O reducing isolates were between 6-8 and 15-35°C [27-30], respectively. Out of these ranges the N₂O reducing activities were obviously restricted. However, the lack of knowledge about the mechanisms of differential functional performances of N₂O reducers would limit our understanding of the shifts of N₂O reducing communities in ecosystems.

As the rapid development of sequencing techniques and bioinformatics in recent years, it is applicable to reveal metabolic pathways and regulating systems by genomic informatics of microbial strains [31]. Therefore, analysis of the genomic characteristics of microorganisms would be curial in understanding their environmental adaptabilities and functions [32]. It was suggested that the activity of N₂O reductase of *nosZ*-containing bacteria could be predicted by the analysis of their structural genes of *nos* clusters, such as *nosR*, *nosD*, *nosF*, *nosY*, *nosL*. [33]. Besides, the transcriptional regulators would also play important roles in manipulation of the functional activity of N₂O reductase, like NarX, NarL, FNR, DNR and so on [34]. So, the genomic characteristics and the regulation system of *nos* operons could be important in explaining the differences of N₂O reducing abilities between N₂O reducers.

In this study, two *nosZ*-containing isolates named *P. veronii* DM15 (DM15) and *P. frederiksbergensis* DM22 (DM22) were selected, and their N₂O reducing abilities, *nosZ* gene expression and whole genome sequences were systematically determined. The objectives were to explore the mechnisms of the differential N₂O reducing abilities of the selected strains. The results would broaden our knowledge in understanding the community function of N₂O reducers.

2. Materials and methods

2.1 Isolation and identification of N₂O reducers

A paddy soil (0-15 cm) was collected for denitrifying bacteria isolation in Jiaying (N: 30°38'21.6", E: 120°46'50.9") in Dec 2014, it had been used for rice production for centuries. Soil denitrifiers were isolated by using enrichment medium (K₂HPO₄ 0.5 g/L, MgSO₄·7H₂O 0.2 g/L, Na₂HPO₄·12H₂O 10.5 g/L, KNO₃ 2 g/L, succinic acid 0.52 g/L). Solid medium was made by adding 15 g agar per liter liquid

enrichment medium. Fresh soil (20 g) was added to a 250 mL conical flask with 20 mL sterile water and then incubated for ten days under 30°C and 120 rpm followed by three days without shaking, this process was repeated three times. Afterwards, 1 mL upper solution was transferred to a new sterile flask containing 30 mL fresh liquid medium and incubated for 24 h at 30°C and 120 rpm and 100 µL of the suspension was plated onto solid medium and cultured overnight at 30°C, then single colonies was transferred onto a solid medium plate with streaking successively three times to get pure strains.

To screen N₂O reducers, the genomic DNA of the above isolates was extracted from 2 mL suspension when the isolates were cultured to the logarithmic stage (after about 12 hours of culturing), using the TIANamp Bacterial DNA Kit. *nosZ* gene fragment were amplified and sequenced with *nosZF/nosZR* primers[35]. Then preliminary functional experiment of N₂O reduction of the isolates were conducted by cultivation with 179.59 µg/L N₂O and detection of N₂O consumption. Two N₂O reducers named *P. veronii* DM15 (DM15) and *P. frederiksborgensis* DM22 (DM22) were selected for experiment material in this study.

2.2 Cell preparation and sampling

Cell culture experiments were designed to test the functional behaviors of the strains under varied oxygen and temperature conditions, the oxygen concentrations were 0%, 10%, 21% O₂ at 30°C and the temperatures were 20°C, 30°C, 40 °C under 0% O₂. The cell culture was conducted with inoculation of DM15 and DM22 independently, while a no inoculation control was set for each experiment. All the treatments had three replications. The detailed procedures are as follows:

Firstly, the Erlenmeyer flasks containing 30 mL denitrification medium (DM) inoculated with DM15 and DM22 independently were cultivated for 24 h at 30°C and 150 rpm. This DM was prepared as follows[36]: K₂HPO₄ 0.5 g/L, NaH₂PO₄ 0.25 g/L, MgSO₄·7H₂O 0.2 g/L, KNO₃ 2 g/L, trisodium citrate 11.35 g/L (C/N=10), trace element stock solution 500 µL/L, pH value 7.0. Afterwards, 1 mL above broths was transferred into a culture bottle with 30 mL DM respectively and the bottles were tightly sealed with butyl rubber stoppers and a crimped metal seal. After about 16 h cultivation (30°C and 150 rpm) when the cell growth was at logarithm stage and OD₆₀₀ value reached above 0.5, the cells were collected by centrifuging at 4000 rpm for 10 min. The pellet was resuspended in 30 mL KNO₃-free DM followed by twice washes with this solution. Then the OD₆₀₀ (Bioscreener C, FP-1100-C) of the suspension was adjusted to 0.5 with KNO₃-free DM for each culture and transferred 30 mL adjusted suspension into a 100 mL culture bottle.

The sealed bottles were vacuumized and 179.59 µg/L N₂O gas was immediately filled through a 0.22 µm sterile filter, then were incubated for 10 min at 150 rpm. For temperature experiment, the incubation was conducted at 20, 30, 40°C under anaerobic condition, independently. For oxygen experiment, the oxygen concentration was controlled as 0%, 10%, 21% by filling the bottle space with oxygen and helium.

Cell sampling for RNA analysis was carried out by taking 1 mL cell suspension before and after the 10 min incubation for all the treatments, the cell samples were flash frozen in liquid nitrogen immediately and total RNA was extracted within 12 h, the RNA samples were stored at -80°C. Gas sampling for residue N₂O concentration determination was performed immediately after the 10 min incubation, 20 mL gas sample from the upper space of each bottle was collected and stored in a 12 mL vial (Labco limited high Eycombe, UK).

2.3 Determination of N₂O reducing abilities

N₂O concentration was measured by a gas chromatograph (Agilent 7890A, USA). N₂O reducing

activity was determined by testing the rates of N₂O reduction of the strains and the rates of N₂O reduction calculated by the following formula:

$$\text{N}_2\text{O reduction rate (ng N}_2\text{O/10}^8\text{ cells/min)} = \frac{(\text{Cck}-\text{CIn}) \times 44 \times 100}{22.4} \times \frac{273}{273+T} \div \frac{\text{Ycount} \times V}{10^8 \times 10}$$

In this formula, Cck and CIn were the N₂O concentrations measured in the no inoculation control and inoculation treatment, respectively. T was gas measured temperature. Ycount and V represented the number of cells and the broth volume in incubation, respectively.

Subsequently, the count the number of cells corrected by using flow cytometry (Beckman MoFlo XDP), then drew a standard curve based on the OD600 value and number of cells and the formula followed as:

$$\text{DM15: Y(counts/}\mu\text{L)} = 2.37\text{E}+07 \times \text{OD value} - 1.35\text{E}+06 \text{ (R}^2=0.99\text{)}$$

$$\text{DM22: Y(counts/}\mu\text{L)} = 2.30\text{E}+07 \times \text{OD value} - 1.30\text{E}+06 \text{ (R}^2=0.99\text{)}$$

2.4 Quantification of *nosZ* transcripts

Total RNA of the cultured cells was extracted with the TransZol Up Plus RNA Kit (Transgen, China) according to manufacturer's protocol. Then 1 μg total RNA was used for cDNA synthesis with the TransScript One-Step gDNA Removal and cDNA Synthesis SuperMix Kit (Transgen, China), the reverse transcription was carried out with the specific primer of *nosZ*R [35]. The qPCR reaction was performed in triplicate in a total reaction volume of 10 μL containing 5 μL SYBR green mix I, 0.2 μL Rox (Takara, Dalian, China), 0.2 μL (10 mmol/L) of both forward and reverse primers (*nosZ*1126F: 5'-GGG CTB GGG CCR TTG CA-3' / *nosZ*1381R: 5'-GAA GCG RTC CTT SGA RAA CTT G-3')[8], 1 μL of template cDNA, and ddH₂O was added to 10 μL. Thermal program was run on the ABI prism 7900 system (Applied Biosystem, USA) following conditions: 95°C, 2 min; 40 cycles of 95°C for 15 s, 60°C for 30 s, 72°C for 30 s; 40°C for 30 s. Standard curve was generated using the plasmid containing the fragment of *nosZ* gene and conducted the vitro transcription using the Thermo Scientific TranscriptAid T7 High Yield Transcription Kit (Thermo Scientific). Standard curves were linear (R²= 0.997) in the range used and amplification efficiency was 98% for *nosZ* gene.

2.5 Analysis of the complete genome sequences

Firstly, the genome DNA of DM15 and DM22 was quantified by TBS-380 fluorometer (Turner BioSystems Inc., Sunnyvale, CA), and sequenced using an IlluminaHiSeq 2000 genomic sequencer at Majorbio (Shanghai, China). For Illumina sequencing, the DNA samples were sheared into 400-500 bp fragments using a Covaris M220 Focused Acoustic Shearer, Illumina sequencing libraries were prepared from the sheared fragments using the NEXTflex™ Rapid DNA-Seq Kit. The prepared libraries were used for paired-end Illumina sequencing (2 × 150 bp) on an IlluminaHiSeq X Ten. The data generated from Illumina platform were used for bioinformatics analysis. For the assembly and annotation, all the analyses were performed using I-Sanger Cloud Platform (www.i-sanger.com) from Majorbio. An assembly of the clean reads were performed using SOAPdenovo2. For Gene prediction and annotation, Glimmer was used for CDS prediction, tRNA-scan-SE was used for tRNA prediction and Barrnap was used for rRNA prediction (<https://github.com/tseemann/barrnap>). The predicted CDSs were annotated from NR, Swiss-Prot, Pfam, GO, COG and KEGG database using sequence alignment tools such as BLAST, Diamond and HMMER (<http://eddylib.org/software/hmmer3/3.1b2/Userguide.pdf>). Average Nucleotide Identity (ANI) is a measure of nucleotide-level genomic similarity between the coding regions of two genomes. The ANI Calculator uses the OrthoANIu algorithm, the ANI value results is

obtained through the website (<https://www.ezbiocloud.net/tools/ani>). Additionally, an effective method of examining synonymous codon usage bias is the codon adaptation index (CAI) value[37], which was calculated with the EMBOSS V6.6.0.0 software. The codon usage table using the Epsesm.cut (Species name: *Pseudomonas syringaepvtomatostr* DC3000), and the path of codon database was EMBOSS-6.6.0/emboss/data/CODONS.

2.6 Statistical analysis

One-way analysis of variance (ANOVA) was used to assess the significant difference in the N₂O reducing rates and abundance of *nosZ* transcripts under various temperature and oxygen concentrations. Pearson correlation analysis was employed to quantify the linear relationships between the N₂O reducing rates and its *nosZ* transcript copies. ANOVAs and Pearson correlation analysis were performed with SPSS software (SPSS Inc., Chicago, IL). The least significant difference (LSD) was used for multiple comparisons among treatments. Statistical significance was determined at $p < 0.05$.

3 Results

3.1 Morphological and General genomic features

Morphologically, both DM15 and DM22 possessed smooth colony surface and white with slight yellowish color. Meanwhile, both strains were rod-shaped, lacking flagella and non-spore-forming tested with scanning electron microscopy (Fig.S1). In addition, the complete genome sequence of the two strains were deposited at DDBJ/ENA/GenBank under the accession number JAARMA000000000 and JAAUOE000000000. Genomic features of DM15 and DM22 were summarized in Table 1. The ANI (Average Nucleotide Identity) value showed that the similarity between the genomic sequences of the strains was 81.72%. According to the sequence data, DM15 contained 6,646,098 total base pairs with GC content of 60.69%, while DM22 possessed 5,957,529 bp with 59.57% GC content. The predicted gene numbers of DM15 were 6018, while that of the DM22 were only 5386.

3.2 N₂O reducing abilities

Temperature significantly influenced the N₂O reducing capabilities of both DM15 and DM22 strains under anaerobic condition (Fig. 1A). The results showed that DM15 possessed significantly stronger N₂O reducing activity than DM22 throughout all the testing temperatures. The maximum differences appeared at 20°C while the N₂O reduction rate of DM15 was about 5.3 times higher than DM22, and the minimum variances occurred at 30°C while the difference reduced to 1.6 times. More interestingly, the function of these two N₂O reducers differently responded to the changes of temperature, the N₂O reduction rate of DM15 were positively related to temperature, it significantly increased 86% from 20°C to 30°C and slightly increased from 30°C to 40°C without significance. However, DM22 showed a differed pattern, its rate of N₂O reduction was very low at 20°C with 3.6 ng N₂O/10⁸ cells·min⁻¹, when temperature was increased from 20°C to 30°C, the rate raised from 3.6 to 21.75 ng N₂O/10⁸ cells·min⁻¹, but it quickly decreased to 12.38 ng N₂O/10⁸ cells·min⁻¹ at 40°C. Therefore, based on the functional responses of DM15 and DM22 towards temperature, the former expressed strong N₂O reducing activities under the testing temperatures.

The N₂O reduction rates of DM15 and DM22 responded to the change of O₂ levels were observed (Fig. 1B). It was detected that the highest N₂O reducing capacities of both DM15 and DM22 appeared under anaerobic, which were 32.39 and 21.40 ng N₂O/10⁸ cells·min⁻¹, respectively, the former was significantly higher than the later. Although these strains belong to the same genus, they possessed varied strategies towards increasing oxygen environment. The N₂O reducing activity of DM15 was much more

sensitive to low level (10%) of O₂, it steeply dropped approximately 85% compared to anaerobic condition, while DM22 only reduced about 60%. Further increasing O₂ to 21% caused continuous decrease of N₂O reducing activity of both strains, while the rates of DM15 and DM22 dropped 33% and 83% the later almost lost its N₂O reducing function under aerobic environment.

3.3 Abundance of *nosZ* transcripts

The results indicated that the copy numbers of *nosZ* transcripts of DM15 and DM22 were obviously affected by temperature (Fig. 2A). The abundance of *nosZ* transcripts of DM15 was 3.94, 3.35, 2.50 folds higher than that of DM22 at 20°C, 30°C, 40°C, respectively. Both strains exhibited that their highest expressions of *nosZ* occurred at 30°C, which were significantly more than that at 20°C and 40°C, correspondingly, except for DM22 between 30°C and 40°C. The abundance of *nosZ* transcripts of either DM15 or DM22 at 20°C was the lowest.

It was also observed that the expression of *nosZ* gene in both DM15 and DM22 were seriously inhibited by the increasing oxygen (Fig. 2B). The abundance of *nosZ* transcripts of both strains was on their top levels under anaerobic condition, but DM15 had significantly higher *nosZ* transcripts (2.06E+07 copies per µg RNA) than that of DM22 (8.32E+06 copies per µg RNA). Compared to DM22, the inhibiting effect of 10% O₂ on the expression of *nosZ* of DM15 was much more severe, its abundance dropped 74.8% whereas the former only decreased 33.1% but there was no significant difference between the strains. Further exposed to 21% O₂, the *nosZ* transcripts of DM15 did not shift obviously in comparison with that under 10% O₂, but it was decreased about 74.9% in DM22, indicating DM22 was more sensitive to aerobic condition.

Furthermore, the N₂O reducing activities of DM15 were not significantly correlated to its *nosZ* transcript copy numbers on temperature regime ($p=0.657$), but significantly correlated to that under oxygen treatments ($p=0.966^{**}$). This ability of DM22 was significantly related to the expression of *nosZ* gene under both temperature and oxygen testing regimes ($p=0.804^{**}$, $p=0.896^{**}$).

3.4 Functional genes annotation

All putative protein coding sequences were searched against the COG databases (clusters of orthologous groups of proteins). Among the 25 COG categories, the gene sequences of DM15 and DM22 were annotated into 21 categories (Fig. 3), and the former generally consisted of more gene numbers in all the categories than the later. Among the categories, the gene numbers of several categories related to transcription and metabolisms indicated that DM15 contained obviously more than DM22, such as DM15 had 104 more gene numbers of [K] (transcription), 45 more of [E] (amino acid transport and metabolism) and 7 more of [J] (Translation, ribosomal structure and biogenesis) than DM22. It was suggested that DM15 may have the advantages in transcription and translating process.

3.5 Gene compositions related to nitrogen metabolism

The genes of DM15 and DM22 were annotated linked to nitrogen metabolism pathways based on KEGG analysis (Fig. 4). It exhibited that the compositions of nitrogen metabolism related genes were similar between them. Firstly, both DM15 and DM22 contained *narG/H/I/J*, *nirB/D*, and *nasA/B* for the dissimilatory/assimilatory nitrate reduction to promote the conversion of nitrate to nitrite or ammonia. Secondly, they carried the complete denitrification genes, including *narG/H/I*, *nirS*, *norB/C* and *nosZ*, indicating that both strains were typical denitrifying bacteria. In addition, both DM15 and DM22 had no nitrogen-fixing genes, and the genes involved in nitrification contained only *norA*. It suggesting that the composition of nitrogen cycling genes in these N₂O reducers was similar.

3.5 Gene compositions of *nos* clusters and predicted *nos* gene expression potentials

The annotation information of the genes in the *nos* regions of DM15 and DM22 was listed in Fig. 5A & B. The expressivity of N₂O reductase was indirectly assessed by calculating the CAI values of the genes in *nos* cluster (Fig. 5C). The *nos* regions of DM15 and DM22 similarly comprised six functionally assigned genes, including *nosR*, *nosZ*, *nosD*, *nosF*, *nosY* and *nosL*. Additionally, the FNR binding site and promoter were both predicted in their *nos* cluster. What the difference was, a *dnr* gene was detected in the *nos* region of DM15 but not in that of DM22. DNR, an FNR-related transcriptional regulator for the expression of denitrification genes. Comparing to the CAI values of the reference gene of *rpsR*, the *nos* genes of both DM15 and DM22 were predicted with high expression potentials. The CAI values indicated that the *nos* genes of DM15 possessed obviously higher expressivities than that of DM22, especially for the *nosZ* gene, the CAI value of DM15 was 0.831, which was the highest and about 6% higher than DM22.

4. Discussion

Among soil denitrifiers, *Pseudomonas* sp. are testified as typical denitrifying bacteria in various environments [38]. Although previous studies revealed that *Pseudomonas* strains showed different nitrate removal efficiencies in wastewater and bioreactors [25, 39], and *nosZ* gene expression could be influenced by environmental factors [16, 17, 29, 40], little was known about the functional difference and the driving mechanisms between N₂O reducers.

In the present study, it was observed that although DM15 and DM22 belong to the same genus of *Pseudomonas*, they performed significantly different N₂O reducing abilities. Since it has been reported that environmental factors could lead to the functional differentiations of denitrifying bacteria, such as temperature, oxygen level, pH and C/N [23, 26, 29, 30], whether the functional difference of these strains was related to environmental conditions? The results of the present study indicated that either under anaerobic and aerobic or varied temperature conditions, DM15 regardless performed higher N₂O reducing ability than DM22, which suggested that environmental factors would not be the main reason for their differential N₂O reducing abilities. Thus, the functional differentiation of DM15 and DM22 would be linked to their inner regulations systems.

Based on genomic characters, several possibilities would be linked to N₂O reduction, such as the gene compositions of nitrogen cycling, *nos* operons and their regulation systems [41, 42]. The gene composition involved in nitrogen cycling could be important and the difference of other genes compositions in denitrification would influence N₂O reduction due to variance of the substrate's concentration [43, 44]. It was detected in the present study that the genes composition of DM15 in the nitrogen cycle, including the dissimilatory /assimilatory nitrate reduction, denitrification, and all other related processes (Figure 4), was the same as DM22, which indicated that the nitrogen cycling genes composition might not be the main force to induce their function difference and it's more likely to be linked the *nosZ* gene transcriptional regulation.

Normally, an operon consists of promoter(s), structural genes and regulator genes. It was observed in this study that DM15 and DM22 contained the same composition of *nos* structural genes in their *nos* cluster regions [33, 45], but different regulation systems. The difference was that the regulator gene of *dnr* presented in the *nos* cluster of DM15 but not in DM22. Since *dnr* encodes DNR which was the Crp/Fnr family transcriptional regulator, it can regulate the *nos* genes transcription and activate the *nosR* promoter [34, 46]. So, the presence of *dnr* gene in the *nos* cluster of DM15 would suggest that the expression of its *nosZ* gene could be stronger. Actually, the advantage of transcriptional related genes

and the actual abundance of *nosZ* transcript also provide the evidences for supporting the above speculation. Additionally, the prediction of *nos* genes expression by CAI values [47] further indicated that DM15 possessed the stronger expressed potential than DM22. Thus, the existence of *dnr* gene in the *nos* cluster of DM15 would be strongly linked to the stronger N₂O reducing abilities in comparison with DM22.

In conclusion, the strains of *P. veronii* DM15 and *P. frederiksbergensis* DM22, exhibited obviously different N₂O reducing abilities, and the transcriptional regulation of *nos* gene region would play a significant role in explaining these differences of N₂O reducing abilities of the two N₂O reducing isolates.

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AUTHORS' CONTRIBUTIONS

Wenxue Wei, Rong Sheng and Chunmei Liu planned and designed the research; Chunmei Liu and Xing Chen performed the bio-assays; Chunmei Liu and Yi Liu analyzed the genomic data; Chunmei Liu interpreted the data and wrote the manuscript; Wenxue Wei and Rong Sheng modified the manuscript.

Conflict of Interest

The authors declare no competing interests.

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TABLE:

Table1 General features of genomic information of DM15 and DM22

FIGURES:

Figure 1 The reducing N₂O reduction rates of *P. veronii* DM15 and *P. frederiksbergensis* DM22 affected by temperatures under anaerobic (A) and different oxygen concentrations at 30°C (B). Data represented in mean ± standard deviation (n = 3) and small letters showed significance at $p < 0.05$ level.

Figure 2 The abundance of *nosZ* transcripts affected by temperatures under anaerobic (A) and different oxygen concentrations at 30 °C (B). Different letters represent significant differences at $p < 0.05$ among treatments (n=3).

Figure 3 The gene numbers in COGs categories of *P. veronii* DM15 and *P. frederiksbergensis* DM22.

Figure 4 Composition of nitrogen cycling related genes of *P. veronii* DM15 (red solid box) and *P. frederiksbergensis* DM22 (blue dotted box) based on the KEGG analysis.

Figure 5 Genome maps of *nos* clusters *P. veronii* DM15 (A) and *P. frederiksbergensis* DM22 (B), and the CAI values (C) of *nos* cluster of the two *Pseudomonas* strains with *rpsR* gene as reference.

SUPPLEMENTARY MATERIAL:

Figure S1 Scanning electron microscopy of strains (10 000 ×). DM15: *P. veronii* DM15; DM22: *P. frederiksbergensis* DM22.

Figures

Figure 1

The reducing N_2O reduction rates of *P. veronii* DM15 and *P. frederiksborgensis* DM22 affected by temperatures under anaerobic (A) and different oxygen concentrations at 30°C (B). Data represented in mean $\pm$ standard deviation (n = 3) and small letters showed significance at $p < 0.05$ level.

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