Environmental Pollution 239 (2018) 43–52

Contents lists available at ScienceDirect

Environmental Pollution

journal homepage: www.elsevier.com/locate/envpol

Gene cloning, expression, and reducing property enhancement of nitrous oxide reductase from *Alcaligenes denitrificans* strain TB☆

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ARTICLE INFO

Article history:
Received 4 December 2017
Received in revised form
14 March 2018
Accepted 1 April 2018

Keywords:
Nitrous oxide reductase
Alcaligenes denitrificans
Gene cloning
Gene expression
Reducing property enhancement

ABSTRACT

Nitrous oxide (N_2O) is a potent greenhouse gas and tends to accumulate as an intermediate in the process of bacteria denitrification. To achieve complete reduction of nitrogen oxide (NO_x) in bacteria denitrification, the structural gene *nosZ* encoding nitrous oxide reductase (N_2OR) was cloned from *Alcaligenes denitrificans* strain TB (GenBank JQ044686). The recombinant plasmid containing the *nosZ* gene was built, and the expression of *nosZ* gene in *Escherichia coli* was determined. Results show that the *nosZ* gene consisting of 1917 nucleotides achieves heterologous expression successfully by codon optimization strategy under optimal conditions (pre-induction inoculum OD_{600} of 0.67, final IPTG concentration of 0.5 mM, inducing time of 6 h, and inducing temperature of 28 °C). Determination result of gas chromatography confirms that N_2O degradation efficiency of recombinant *E. coli* is strengthened by at least 1.92 times compared with that of original strain TB when treated with N_2O as substrate. Moreover, N_2OR activity in recombinant strain is 2.09 times higher than that in wild strain TB, which validates the aforementioned result and implies that the recombinant *E. coli* BL21 (DE3)-pET28b-*nosZ* is a potential candidate to control N_2O accumulation and alleviate greenhouse effect. In addition, the N_2OR structure and the possible N_2O binding site in *Alcaligenes* sp. TB are predicted, which open an avenue for further research on the relationship between N_2OR activity and its structure.

1. Introduction

Nitrous oxide (N_2O) is a small but potent greenhouse gas. It accounts for only 10% of total greenhouse gas emissions, but its global warming potential is 298 times higher than that of carbon dioxide (Black et al., 2016; Mampaey et al., 2016). The atmospheric concentration of N_2O relative to air has increased by almost 20% in the last 100 years, and the majority of N_2O emission into the atmosphere is produced by microbial metabolism of nitrogen compounds, such as NO, in soils and oceans (Pauleta et al., 2013). Human activities also contribute to its emission, mainly including the usage of fertilizers, application of livestock manure in crop lands, and fuel combustion (Lassey and Harvey, 2007). The microbial metabolism of nitrogen compounds is mainly divided into nitrification and denitrification. N_2O is an intermediate product in the denitrification process: $NO_3^- \rightarrow NO_2^- \rightarrow NO \rightarrow N_2O \rightarrow N_2$. Bacteria denitrification, the final step of anaerobic respiration of nitrate, can reduce N_2O to molecular nitrogen (N_2), and its reduction represents the loss of biologically available nitrogen.

In industry, N_2O emission reduction technologies mainly include catalytic direct decomposition and catalytic reduction (Lee et al., 2012). A nitric acid plant in Bulgaria has conducted a project for the catalytic decomposition of N_2O, resulting in a total N_2O emission reduction of approximately 6614 Mg in 75 months compared to the control group (Stefanova and Chuturkova, 2014). In wastewater treatment, denitrifying bacteria and immobilized *Alcaligenes faecalis* gels were introduced to reduce N_2O emissions through proper operation and management (Readdaily et al., 2016; Young et al., 2007). Appropriate supplement of influent Cu^{2+} is beneficial to reduce N_2O emission during biological nutrient removal from municipal wastewater (Zhu et al., 2013). In agriculture and livestock farming, biochar can be used to mitigate greenhouse gas emissions from soils, especially N_2O, as confirmed by several studies (Ameloot et al., 2016; Hagemann et al., 2017;

☆ This paper has been recommended for acceptance by Dr. Harmon Sarah Michele.

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https://doi.org/10.1016/j.envpol.2018.04.005
0269-7491/

Ramlow and Cotrufo, 2018). N_2O mitigation can also be achieved by reasonably reducing the use of nitrogen fertilizers or applying a nitrification inhibitor (Wu et al., 2017b; Zhang et al., 2018), and the $N_2O/(N_2O + N_2)$ product ratio decreased with the decrease of nitrate concentration in the soil, indicating that the decrease of nitrate promoted the reduction of N_2O to N_2 (Qin et al., 2017).

N_2O reduction in the natural environment is an enzymatic reaction that can only be catalyzed by nitrous oxide reductase (N_2OR) using N_2O as substrate. High N_2O emission in the environment is generally associated with N_2OR or its encoding gene-missing/lacking denitrifying bacteria (Ma et al., 2017; Nie et al., 2015, 2016). Therefore, in-depth exploration of the characteristics of N_2OR and its gene is of crucial importance to mitigate N_2O emission.

N_2OR, the terminal enzyme of denitrification, is a multi-copper protein encoded by a *nos* gene cluster (*nosRZDFYL*). It has been isolated and biochemically characterized from different classes of *Proteobacteria*. The first active N_2OR was characterized from *Pseudomonas stutzeri* (classified at the time as *P. perfectomarinus*) (Zumft and Matsubara, 1982), and the enzyme structure was resolved in several bacteria such as *Marinobacter hydrocarbonoclasticus* (formerly *Pseudomonas nautica*) (Brown et al., 2000b), *Paracoccus denitrificans* (Haltia et al., 2003), *Achromobacter cycloclastes* (Paraskevopoulos et al., 2006) and *P. stutzeri* (Pomowski et al., 2011). The overall folding of N_2OR is similar in all the solved structures. It is a head-to-tail homodimer. Each monomer contains two domains: an electron transferring domain (binuclear CuA center) and a catalytic domain (tetranuclear CuZ center). But there is some difference in the elaboration about CuZ bridging and cupric coordination in the catalytic CuZ center, resulting in different prediction patterns of substrate binding. The catalytic mechanism of N_2O reduction in N_2OR is still unclear. The structural gene of N_2OR, *nosZ*, has been previously sequenced for several different organisms (Hoeren et al., 1993; Holloway et al., 1996; Velasco et al., 2004), and it can be used as a target for different populations of denitrifying bacteria capable of N_2O reduction (Throbäck et al., 2004). In *P. stutzeri*, the transcription of *nosZ* was dependent on *nosR* gene (Pauleta et al., 2013). *nosDFY* putatively encode an ABC transporter system for the biogenesis of CuZ center (Zumft and Kroneck, 2006), and the catalytically active holoenzyme cannot be obtained when only *nosZ* but lacking *nosDFY* was expressed in a heterologous host (Wunsch et al., 2003). It has been reported that a homologous expression system containing the *nos* gene cluster from *A. cycloclastes* was developed and a functional recombinant N_2OR, which may contain CuA and CuZ centers, was obtained by anaerobic purification (Fujita et al., 2007). Recently, the *nosZ* gene from *Geobacillus thermodenitrificans* was expressed in *E. coli*, producing active, albeit copper-deficient, recombinant N_2OR (Liu et al., 2008). Moreover, tobacco plants were engineered to express the single-gene *nosZ* and multi-gene *nosFLZDY*. The results showed that the products of other *nos* genes may not be necessary for the expression and assembly of N_2OR in plants (Wan et al., 2012b). Transgenic plants expressing active N_2OR could conceivably play a positive role in alleviating N_2O emissions (Wan et al., 2012a, 2014).

One denitrifying bacterium identified as *Alcaligenes denitrificans* strain TB, which was screened out from a rotating drum biofilter, was investigated. In this work, the *nosZ* gene was cloned with the extracted genomic DNA as template from strain TB by polymerase chain reaction (PCR) amplification with a pair of specific primers. In addition, a 1917 bp DNA sequence was optimized for better protein expression and was inserted into the pET28b vector and subsequently expressed in *Escherichia coli* to obtain recombinant strain *E. coli* BL21 (DE3)-pET28b-*nosZ* with stronger N_2O reduction ability. This recombinant *E. coli* holds promise for controlling N_2O accumulation and alleviating greenhouse effect.

2. Materials and methods

2.1. Materials

Strains and plasmids. *Alcaligenes denitrificans* strain TB was isolated from the biofilm of a multi-layer RDB used for NOx removal from simulated flue gas (Chen et al., 2016).

E. coli BL21 (DE3) and vector pGEM-T and pET28b were purchased from Novagen (USA).

Media. TB and *E. coli* strains were grown aerobically in L-broth medium as follows (per liter): NaCl, 10.0 g; yeast extract, 5.0 g; peptone, 10.0 g; and agar, 20.0 g at pH 7.2.

Chemicals and enzymes. DNA marker and PCR primers were bought from Sangon Biotech Co. (Shanghai, China). Gene was sequenced at Sangon Biotech Co. *Xba* I and *Xho* I were bought from Shanghai Bioleaf Biotech Co., Ltd., China. Kanamycin and IPTG were obtained from Beijing Kangwei Biotechnology Co., Ltd., China.

2.2. PCR amplification

The genomic DNA of *Alcaligenes* sp. TB was used as DNA template for PCR. Total genomic DNA was extracted according to the instructions of a DNA isolation kit provided by Shanghai Biotechnology Co., Ltd. Afterward, DNA was stored at 4 °C.

PCR was performed in a Mastercycler gradient (Bio-Rad, USA) by using the following primers: GW003-NF (5′-ATGTCCGACAAGAATCCCGA-3′) and GW003-NR (5′-CTACGCGGTCTTCTTCGGGT-3′). PCR mixture (20 μL) consisted of 2 μL 10 × DreamTaq Buffer, 0.5 μL dNTPs (10 mM), 1 μL oligonucleotide primers, 0.2 μL Taq enzyme, 1 μL template DNA, and 14.3 μL deionized water. The mixture was denatured at 94 °C for 5 min, followed by 32 cycles of predenaturation at 94 °C for 5 min and denaturation at 94 °C for 0.5 min; annealed at 57 °C for 0.5 min; extended at 72 °C for 2 min; and subjected to a final extension at 72 °C for 7 min. After amplification and purification, the PCR products (*nosZ* gene) were sequenced.

The DNA sequences were translated into amino acid sequences using DNAMAN. The amino acid sequences were aligned by BLAST, and a phylogenetic tree was constructed by neighbor-joining method with 1000 bootstrap trials by using MEGA v7.0.26 software. Meanwhile, a three-dimensional protein structure was predicted using an online website (https://www.swissmodel.expasy.org/) and then edited in the Discovery Studio Visualizer 3.5 software. The template protein used for modeling was N_2OR from *P. denitrificans* (PDB ID: 1fwx). The distances between the copper centers and the distances between the atoms were calculated based on DS Visualizer 3.5. Amino acid coordination of the copper centers was constructed according to the “Receptor-Ligand Interactions” tool in the DS Visualizer 3.5.

2.3. Codon optimization

Codon optimization was performed by the online program Optimizer (http://genomes.urv.es/OPTIMIZER) based on the analysis of rare codon (http://nihserver.mbi.ucla.edu/RACC) for better protein expression in *E. coli*. The Genscript Web server (http://www.genscript.com) was used to evaluate the CAI of sequence. The codon-optimized *nosZ* gene was synthesized by Jinhua Bomei Biotechnology Co., Ltd.

2.4. Construction of expression vector

The synthesized *nosZ* sequence was subsequently cloned into the pGEM-T vector, and the *nosZ* was confirmed by *Xba* I and *Xho* I digestion together with direct DNA sequencing. Next, the released