

The *nos* gene cluster from gram-positive bacterium *Geobacillus thermodenitrificans* NG80-2 and functional characterization of the recombinant NosZ

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Abstract

The *nos* gene cluster encoding the activity of nitrous oxide reductase (N₂OR) for the final step of the denitrification pathway has been well studied in gram-negative bacteria. Our previous study on the genome of *Geobacillus thermodenitrificans* NG80-2 revealed the presence of the *nos* gene cluster in this gram-positive bacterium. In this follow-up study, the *nos* gene cluster of *G. thermodenitrificans* NG80-2 was further analyzed and compared with those of other origins. The structural gene *nosZ* was heterologously expressed in *Escherichia coli* and the product was purified as a His-tagged fusion protein. The recombinant NosZ as prepared showed detectable N₂OR activity, and the activity was enhanced by preincubation of the protein under argon and with copper compounds. The recombinant NosZ contains 2.5 atoms of copper per dimer and exhibits weak spectral features in the visible range, indicating that spontaneous incorporation of copper compounds into the NG80-2 NosZ can result in some but not full activity of the authentic NG80-2 N₂OR. The enzymatic properties of the NosZ were also investigated. This is the first functional characterization of *nosZ* gene from gram-positive bacteria. This study indicates that the molecular mechanism for N₂O reduction is conserved between gram-negative and gram-positive bacteria.

Introduction

Denitrification, also known as one example of anaerobic respiration, is a process in which nitrogenous compounds are used as alternative electron acceptors for energy production (Knowles, 1982). In the environment, denitrification contributes to the greenhouse effect and destruction of the ozone layer, and it is also the major process for the return of fixed nitrogen to the atmosphere (Zumft, 1997). Complete denitrification includes four reaction steps (NO₃⁻ → NO₂⁻ → NO → N₂O → N₂) catalyzed by nitrate reductase, nitrite reductase, nitric oxide reductase, and nitrous oxide reductase (N₂OR) sequentially. The process is known to be carried out by members of both gram-negative and gram-positive bacteria (Zumft, 1997; Suharti & de Vries, 2005). Denitrification genes including *nar* (encoding nitrate reductase), *nir* (encoding nitrite reductase), *nor* (encoding

nitric oxide reductase) and *nos* (encoding N₂OR) genes have been well studied, mainly in gram-negative bacteria (Zumft, 1997).

N₂OR, which catalyzes the conversion of N₂O into N₂ in the last step of denitrification, has been isolated and characterized in many gram-negative bacteria (Snyder & Hollocher, 1987; Ferretti *et al.*, 1999; Prudencio *et al.*, 2000; Yamaguchi *et al.*, 2003). It is a homodimeric protein with each subunit containing two multinuclear copper centers, electron-transferring site Cu_A and catalytic site Cu_Z (Zumft & Kroneck, 2007). Depending on the source and purification process, different spectroscopic properties of the protein can be obtained (Rasmussen *et al.*, 2002; Zumft & Kroneck, 2007). In gram-negative bacteria, N₂OR activity is encoded by the *nos* gene cluster, which contains *nosZ* (encoding the catalytic unit of N₂OR), *nosDFY* (encoding an ATP-binding cassette transporter system for assembly of

copper centers) and other accessory genes (Philippot, 2002; Zumft & Kroneck, 2007).

Geobacillus thermodenitrificans is a facultative aerobe, capable of growth by denitrification (Manachini *et al.*, 2000). Recently, we sequenced the whole genome of denitrifying *G. thermodenitrificans* NG80-2, and reported the presence of the *nos* gene cluster as the first such case in gram-positive bacteria (Feng *et al.*, 2007).

In this study, we further compared the NG80-2 *nos* gene cluster with those of other origins. The structural gene, *nosZ*, was heterologously expressed in *Escherichia coli* and functionally characterized *in vitro*. Enzymatic and spectroscopic properties of the NosZ were also investigated.

Materials and methods

Materials and reagents

Primers were synthesized by AuGCT Biotechnology Corporation, Beijing, China. Restriction enzymes and rTaq DNA polymerase were purchased from TaKaRa, T4 DNA ligase from Promega, and DNase I from Roche. Methyl viologen, sodium dithionite and phenylmethanesulfonyl fluoride (PMSF) were purchased from Sigma. Chelating Sepharose Fast Flow column and High Molecular Weight Standards were purchased from Amersham Biosciences. Protein Molecular Weight Marker was purchased from Bio Basic Inc. Other chemicals and reagents were from Shanghai Sangon, China. All the reagents used were of analytical grade.

Bacterial strains and growth conditions

Geobacillus thermodenitrificans NG80-2 and *E. coli* BL21 (DE3) (Novagen) were respectively grown in Luria–Bertani (LB) medium at 60 and 37 °C with shaking. When necessary, 50 mg kanamycin L⁻¹ was added to the medium.

Cloning and plasmid construction

Genomic DNA from *G. thermodenitrificans* NG80-2 was extracted as described previously (Wang *et al.*, 2006). The NG80-2 *nosZ* was amplified by PCR using primers 5'-CGCG GATCCGTGAAGAAAAAAGTGAT-3' and 5'-CCGGAATT CTCATTGTTTCGGCTTCA-3'. PCR was initiated by denaturation at 95 °C for 3 min, followed by 25 cycles of 95 °C for 30 s, 56 °C for 45 s and 72 °C for 2 min, and a final extension at 72 °C for 5 min. The PCR product was digested with BamHI and EcoRI, and the resulting fragment was ligated into pET-28a (Novagen) to generate pLW1192. The presence of the insert in pLW1192 was verified by sequencing using an ABI3730 automated DNA sequencer (Applied Biosystems, Foster City, CA).

Expression and purification

Escherichia coli BL21 carrying pLW1192 was grown in LB medium containing 50 mg kanamycin L⁻¹ to an A_{600 nm} of 0.6. CuCl₂ (0.8 mM) was supplemented, as reported for other copper-containing proteins (Coyle *et al.*, 1985; Viebrock & Zumft, 1988), and expression of the NosZ was induced by 0.1 mM isopropylthio β-D-galactopyranoside (IPTG) at 37 °C for 4 h.

All steps in the following procedures were carried out oxically at 4 °C. *Escherichia coli* cells were harvested by centrifugation at 10 000 g for 10 min, washed with lysis buffer (50 mM Tris-HCl, pH 8.0, 300 mM NaCl and 10 mM imidazole), resuspended in the same buffer containing 1 mM PMSF and 1 g lysozyme L⁻¹, and sonicated (UP200s ultraschallprozessor, Dr Hielscher, 20 kHz, 0.5 cycle, 90% amplitude). Crude extract was obtained by centrifugation at 18 000 g for 30 min, and applied to a Chelating Sepharose Fast Flow column according to the manufacturer's instructions. Unbound proteins were washed out with wash buffer (50 mM Tris-HCl, pH 8.0, 300 mM NaCl and 20 mM imidazole). His-tagged fusion proteins were eluted with elution buffer (50 mM Tris-HCl, pH 8.0, 300 mM NaCl and 250 mM imidazole) and dialyzed against 50 mM Tris-HCl buffer (pH 8.0) containing 20% glycerol.

Protein concentration was determined by the Bradford method (Bradford, 1976). Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and native-PAGE were performed using the methods of Laemmli (1970) and Tulchin *et al.* (1976), respectively.

N₂OR activity assay

N₂OR activity was measured spectrophotometrically at 600 nm under argon by following the N₂O-dependent oxidation of dithionite-reduced methyl viologen (Kristjansson & Hollocher, 1980; Ferretti *et al.*, 1999). The standard reaction mixture (2 mL) contained 50 mM Tris-HCl, pH 9, 0.5 mM methyl viologen, an appropriate amount of the purified NosZ and a suitable amount of 1 mM sodium dithionite to give an A_{600 nm} of 1.0–1.2. The reaction was initiated by injecting 160 μL of N₂O-saturated solution. N₂OR specific activity was defined as micromoles of N₂O reduced min⁻¹ mg⁻¹ protein.

Effects of temperature, pH and EDTA on N₂OR activity

For temperature effect, assays were carried out at pH 9 and variable temperatures of 25–70 °C. For pH effect, assays were carried out at 30 °C and variable pH values ranging of 5–11. The buffers used were citrate–sodium citrate (pH 3.2–5.8), K₂HPO₄–KH₂PO₄ (pH 5.8–8), Tris-HCl (pH 8–9) and glycine–NaOH buffer (pH 9–11). The effect of EDTA was

examined by incubation of the NosZ anoxically in the absence and presence of 0.05, 0.5 and 5 mM EDTA, respectively, for 45 min, before the activity assay.

Determination of copper content

The copper content of the purified NosZ was determined using a 180–80 polarized Zeeman atomic absorption spectrophotometer (Hitachi, Japan).

Bioinformatics methods

Homologous genes were identified by NCBI BLAST search (<http://www.ncbi.nlm.nih.gov/BLAST/>). Amino acid sequences were aligned with CLUSTAL X 1.83 and a phylogenetic neighbor-joining tree was constructed using MEGA 3.1 program. SIGNALP 3.0 (<http://www.cbs.dtu.dk/services/SignalP/>) was used to predict signal peptide regions.

Results

Comparative analysis of the NG80-2 *nos* gene cluster

The NG80-2 *nos* gene cluster comprises seven ORFs in *nosCZ*·*orf*·*nosDYF*·*orf* arrangement (Fig. 1). This arrangement is atypical for *nos* gene clusters, which usually have a conserved *nosRZDFYL* sequence (Chan *et al.*, 1997; Inatomi, 1998; Honisch & Zumft, 2003; Velasco *et al.*, 2004). A similar gene arrangement was found only in *Desulfitobacterium hafniense* (*nosCZ*·*orf*·*nosDLYF*) (Fig. 1). In addition to the structural gene *nosZ* and the essential accessory genes *nosDFY*, *nosC* encoding a cytochrome *c* type protein is also present. Two *orfs* (*GTNG_1733* and *GTNG_1729*) encoding hypothetical proteins are also present. Genes in the NG80-2 *nos* gene cluster are most similar to the corresponding homologues in *D. hafniense* (20–56% DNA identity) except for *GTNG_1729*, the homologue of which is absent in *D. hafniense* (Fig. 1). The closer relationship of the *nos* gene clusters from the two species was further established by phylogenetic analysis of *nosZ* genes (Fig. 2).

The only other *nos* gene cluster with genes encoding cytochrome *c* is in *Wolinella succinogenes* (Simon *et al.*, 2004), and the NG80-2 NosC shows no similarity to *W. succinogenes* NosC proteins apart from the heme *c*-binding motif. The same was found with *D. hafniense* NosZ and NosC. Neither NapG nor NapH homologues were found encoded in the NG80-2 genome. The *nosL* gene encoding a copper chaperone and commonly forming a transcript unit with *nosDFY* is absent in NG80-2. The other described *nos* genes including *nosR* and *nosX* are also absent. No homologues of those *nos* genes were detected in the NG80-2 genome. Similarly, no orthologues of *nosR* or *nosX* were found in *D. hafniense*.

The deduced sequences of NG80-2 NosZ show all conserved ligands of the Cu_A and Cu_Z centers except for the presence of an asparagine residue in place of tryptophan between the two Cu_A cysteine ligands (Fig. 3). Replacement of the tryptophan residue by histidine in *Ralstonia eutropha*, and by glutamic acid in *W. succinogenes*, was also found (see Supporting Information, Fig. S1). The NG80-2 NosZ pre-protein contains a predicted long signal peptide of 37 aa (Fig. S1). However, the two conserved arginine residues (RR-motif), which are commonly found in other NosZ signal peptides and indicative of Tat-dependent exported proteins (Holloway *et al.*, 1996; Philippot *et al.*, 2001), are absent, even though the Tat system composed of Tata (GTNG_0218 and GTNG_1366) and TatC (GTNG_0219), similar to that in *Bacillus subtilis*, is present in NG80-2. Instead, it exhibits some characteristics of Sec-dependent exported proteins, including the presence of positive charged residues in tandem (three lysine residues) at the N-terminal and the A–X–A sequence (ASA) at the C-terminal (Berks, 1996; Tjalsma *et al.*, 2000). *Desulfitobacterium hafniense* NosZ also carries a Sec-type instead of a Tat-type signal peptide as shown by sequence analysis (Simon *et al.*, 2004).

Expression and purification of the recombinant NG80-2 NosZ

The NG80-2 NosZ was expressed as a His-tagged fusion protein in the soluble fraction. The purified NosZ migrated

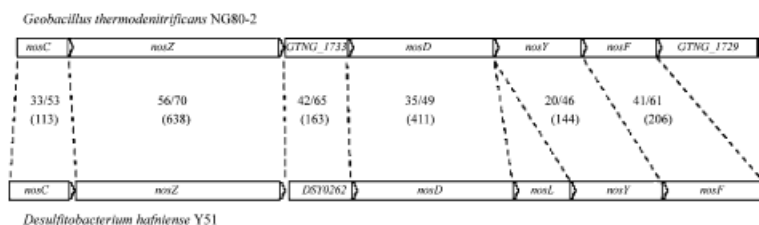


Fig. 1. Comparison of the *nos* gene clusters from *Geobacillus thermodenitrificans* NG80-2 and *Desulfitobacterium hafniense* Y51. All the genes are transcribed in one direction. Dashed lines align homologous genes between NG80-2 and Y51. The number before and after the slash indicates the amino acid sequence identity and similarity of the aligned gene products, respectively, and the number in brackets indicates the number of amino acids overlapping the aligned sequences.

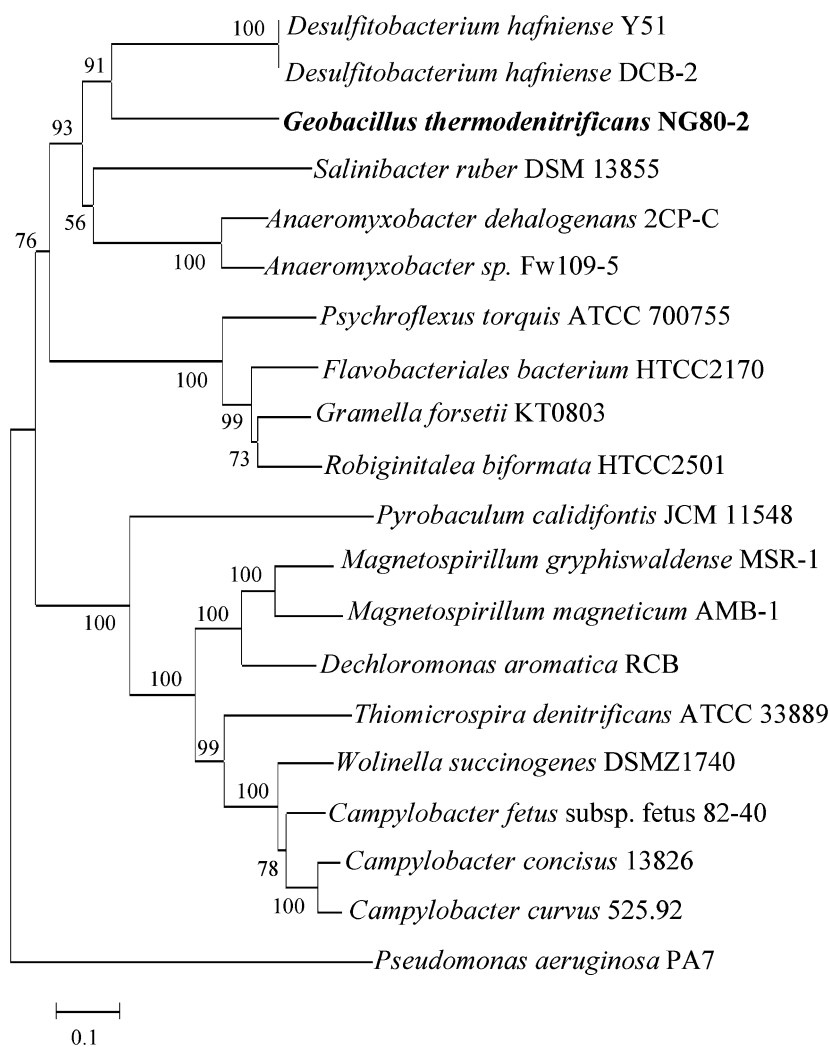


Fig. 2. Phylogenetic tree of NosZ proteins and homologues. The nonredundant protein sequences database of GenBank was searched with the sequences of *Geobacillus thermodenitrificans* NG80-2 NosZ preprotein using BLASTP. The amino acid sequences of the first 20 hits were aligned with CLUSTAL X 1.83 and a phylogenetic neighbor-joining tree was constructed using MEGA 3.1. The numbers on branching points are bootstrap values with 1000 replicates (values of < 50% were not included).

as a band of *c.* 70 kDa in SDS-PAGE (Fig. 3a) and *c.* 140 kDa in native-PAGE gels (Fig. 3b), indicative of a dimer. The subunit of the NosZ estimated to be 70 kDa by SDS-PAGE is in good agreement with the calculated mass of 69 235.3 Da, and within the range for N₂OR subunits of other origins (Zumft, 1997).

Spectroscopic properties and copper content of the recombinant NG80-2 NosZ

The prepared recombinant NG80-2 NosZ was colorless, and gave rise to weak absorption features in the visible range with two recognized bands at 550 and 620 nm (Fig. 4a), corresponding to, respectively, the signatures for the oxidized Cu_Z (*c.* 560 nm) and reduced Cu_Z (*c.* 625–650 nm) reported for other characterized N₂ORs (Rasmussen *et al.*, 2002; Fujita *et al.*, 2007; Zumft & Kroneck, 2007). Additional features were detected at *c.* 670 nm, which was expected to have arisen from Cu_Z^{*}, the redox-inert form of

Cu_Z. Reduction with dithionite anoxically resulted in the formation of 'blue' form enzyme and a spectrum characterized by a single broad band centered at 650 nm (Fig. 4c), in agreement with dithionite-reduced N₂ORs of other origins (Zumft & Kroneck, 2007). Oxidization with ferricyanide anoxically led to the detection of an emerged band at 580 nm, whereas the bands at 550 and 620 nm were either eliminated or decreased (Fig. 4d), indicating the conversion of the 620-nm band and shift of the 550-nm band to the 580-nm band. In addition, absorbance at *c.* 500, *c.* 520 and *c.* 720 nm was also enhanced in the ferricyanide oxidized NosZ, indicating those are signatures for the oxidized Cu_A, which showed similar bands at 480–500, 540 and 760–780 nm as described previously (Yamaguchi *et al.*, 2003; Fujita *et al.*, 2007). The NosZ incubated anoxically for 4 h produced the same spectrum as the ferricyanide-oxidized protein, and the spectrum was enhanced when Cu²⁺ (0.2 mM) was added (Fig. 4b and e). No spectra signals were detected in the enzyme purified from *E. coli*

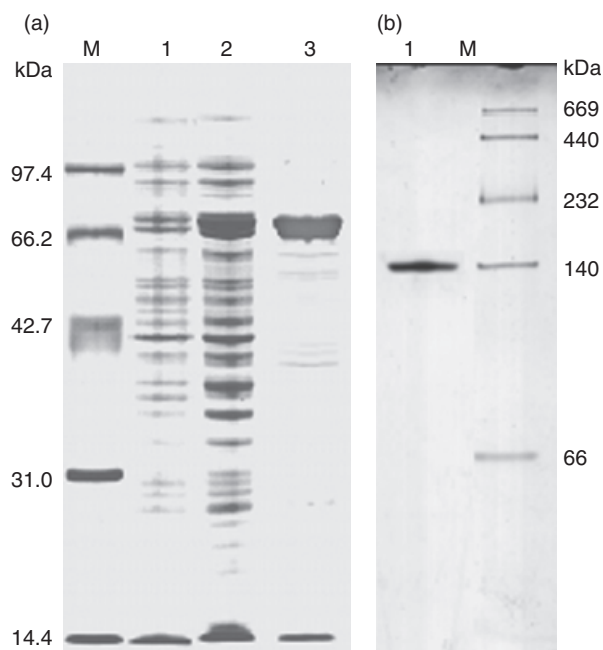


Fig. 3. (a) SDS-PAGE analysis of NG80-2 NosZ expressed in *Escherichia coli* BL21. Lane M, protein molecular weight marker; lane 1, crude extract before IPTG induction; lane 2, crude extract after IPTG induction; lane 3, purified His-tagged NosZ. (b) Native-PAGE analysis of NG80-2 NosZ. Lane 1, purified His-tagged NosZ; lane M, high-molecular-weight standards. To each lane, 3–5 μ g of protein was applied.

cells grown in low copper medium (5 μ M), which contains no copper (Fig. 4f).

The average copper content of the purified NosZ from three independent preparations was 2.5 ± 0.3 atoms per dimer, a value much lower than those reported previously for N₂OR, which range from 7 to 11 atoms per dimer (Snyder & Hollocher, 1987; Ferretti *et al.*, 1999; Prudencio *et al.*, 2000; Yamaguchi *et al.*, 2003). Therefore, there is a low level of spontaneous incorporation of copper into the recombinant NosZ. Some spontaneous copper incorporation was also demonstrated in the recombinant NosZ from *Pseudomonas stutzeri*, but no functional N₂OR was produced (Viebrock & Zumft, 1988).

N₂OR activity of the recombinant NG80-2 NosZ

The prepared recombinant NG80-2 NosZ showed low N₂OR activity, 0.2 ± 0.03 U mg⁻¹ as measured under the optimal assay condition (30 °C, pH 9). The activity increased to 2.9 ± 0.25 U mg⁻¹ when the protein alone was preincubated under argon for 4 h, and to 4.8 ± 0.3 U mg⁻¹ when Cu²⁺ (0.2 mM) was added during the preincubation. The specific activity for the NosZ was similar to the values reported for the N₂ORs from *Thiosphaera panotropha*, *Alcaligenes xylooxidans* NCIMB 11015 and *Pseudomonas perfectomarina* (2–9 U mg⁻¹) (Coyle *et al.*, 1985; Berks *et al.*, 1993; Ferretti *et al.*, 1999),

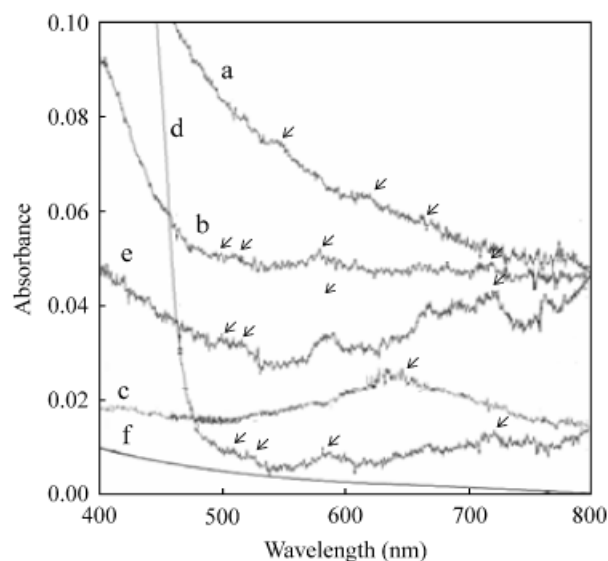


Fig. 4. UV-visible spectra of the recombinant NG80-2 NosZ. (a) As prepared; (b) incubated for 4 h anoxically; (c) reduced with dithionite for 4 h anoxically; (d) oxidized with K₃Fe(CN)₆ for 4 h anoxically; (e) incubated with Cu²⁺ (0.2 mM) for 4 h anoxically; (f) purified from *Escherichia coli* cells grown in low copper medium (5 μ M). The protein concentration was 2 mg mL⁻¹ in 50 mM Tris-HCl, pH 8.0.

but lower than those from *Hyphomicrobium denitrificans* A3151, *Pseudomonas nautica* 617 and *Paracoccus denitrificans* (23–122 U mg⁻¹) (Snyder & Hollocher, 1987; Prudencio *et al.*, 2000; Yamaguchi *et al.*, 2003). The recombinant NosZ prepared from *E. coli* cells cultivated in the presence of a limited amount of CuCl₂ (5 μ M), instead of 0.8 mM used in the standard procedure, showed no N₂OR activity (data not shown), indicating copper is absolutely required for the activity of the protein, as for other reported N₂ORs.

Effects of temperature, pH and EDTA on the activity of the NosZ were also investigated. At pH 9, the highest activity was obtained at 30 °C, then declined to the lowest level at 40–45 °C with around 10% of the activity detected (Fig. 5a). However, as temperature continued to rise, the activity was recovered and a second activity peak with around 65% of the activity was obtained at 55 °C, and 40% of the activity still remained at 70 °C. A similar temperature profile with two peaks was also found for *A. xylooxidans* N₂OR, and this was attributed to the presence of two forms of the enzyme in equilibrium as suggested (Ferretti *et al.*, 1999).

At 30 °C, the activity increased along with a rise in pH values from 5 to 10 when the maximum was reached, and about 80% of the activity remained at pH 11 (Fig. 5b). The effect of EDTA on the enzyme activity was also examined. Under the optimal assay condition, 86%, 77% and 74% of the activity were obtained with the NosZ treated with 0.05, 0.5 and 5 mM EDTA, respectively, compared with non-treated enzyme. Thus, EDTA did not remove copper readily.

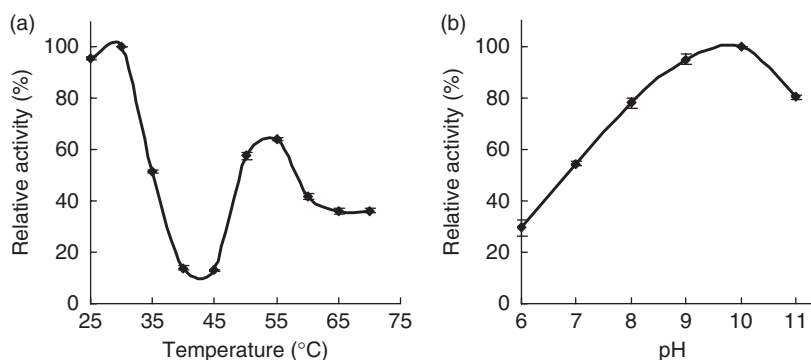


Fig. 5. Effects of temperature and pH on the activity of NosZ. Enzyme activities were assayed at pH 9 in the range 25–70 °C (a), and at 30 °C in the range of pH 6–11 (b). NosZ was preincubated anoxically for 4 h before the assays. The relative activity of 100% corresponds to $2.9 \pm 0.25 \text{ U mg}^{-1}$.

Discussion

Previous study showed that heterologous expression of the NosZ from *P. stutzeri* did not lead to the formation of N₂OR holoenzyme, and this was attributed to the inability to provide functions for the assembly of copper centers by *E. coli* (Viebrock & Zumft, 1988). More recently, a homologous expression system for N₂OR was developed in *Achromobacter cycloclastes*, in which the entire *nos* gene cluster was expressed and functional recombinant N₂OR was obtained under anaerobic conditions (Fujita *et al.*, 2007). In this study, we showed that the NG80-2 NosZ expressed in *E. coli* was still functional but that the enzyme activity was low. Therefore, some copper centers must be assembled in the recombinant NosZ without involvement of other Nos proteins. However, it cannot be determined whether the copper centers are properly assembled, due to the weak spectrum and low copper content. Nevertheless, the function of NG80-2 *nosZ* gene was clearly indicated.

With few exceptions, N₂ORs from gram-negative bacteria are soluble copper-containing proteins residing in the periplasmic space. Gram-positive bacteria have only a very small periplasmic-like space. In *Bacillus azotoformans*, the presence of menaquinol-dependent membrane-bound N₂OR was suggested based on the detection of membrane-associated N₂OR activity (Suharti & de Vries, 2005). However, the enzyme has not been isolated or genetically characterized. In *T. denitrificans*, the membrane-bound N₂OR was shown to be a NosZ homologue (Hole *et al.*, 1996). In NG80-2, the NosZ is also likely to be bound to the membrane to perform the N₂OR function, with the contained Sec-type signal peptide as the membrane anchor. However, this needs to be confirmed experimentally by determining the cellular location of the enzyme in the future studies.

The close relationship between the *nosZ* genes from NG80-2 and *D. hafniense* was expected, as *D. hafniense*, which was originally described as gram-negative based on Gram stain, was later assigned to gram-positive based on phylogenetic analysis of full-length SSU rRNA genes (Niggemeyer *et al.*, 2001). Therefore, the presence of *nos* gene clusters in gram-positive bacteria may be more prevalent,

and remain to be unveiled. The NG80-2 *nos* gene cluster does not contain *nosR*, the regulatory gene commonly present in *nos* gene clusters (Wunsch & Zumft, 2005). The absence of *nosR* was also found for *D. hafniense* and a small group of gram-negative bacteria including *W. succinogenes*. In the latter group, the *nosH* gene was suggested to substitute for the function of *nosR* (Simon *et al.*, 2004). The *nosH* gene was not found in the NG80-2 and *D. hafniense* *nos* gene clusters, and therefore additional genes are expected for this function. The functions of the two unknown *orfs* (*GTNG_1729* and *GTNG_1733*) in the NG80-2 *nos* gene cluster remained to be classified.

In conclusion, we expressed the *nosZ* gene from *G. thermodenitrificans* NG80-2 in *E. coli*, and characterized the gene product as N₂OR. This study indicates that the same molecular mechanism for N₂O reduction is utilized by both gram-negative and gram-positive bacteria.

Acknowledgements

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Alignment of NosZ primary structures from *Geobacillus thermodenitrificans* NG80-2 and other bacteria.

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