Microbiology (2013), **159**, 307–315 DOI 10.1099/mic.0.060194-0

Primers for amplification of nitrous oxide reductase genes associated with Firmicutes and Bacteroidetes in organic-compound-rich soils

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The *nosZ* gene encodes nitrous oxide reductase, a key enzyme in the nitrous oxide reduction that occurs during complete denitrification. Many conventional approaches have used Proteobacteria-based primers to detect *nosZ* in environmental samples. However, these primers often fail to detect *nosZ* in non-Proteobacteria strains, including Firmicutes (Gram-positive) and Bacteroidetes. In this study, newly designed *nosZ* primers successfully amplified this gene from five *Geobacillus* species (Firmicutes). The primers were used to construct *nosZ* clone libraries from DNA extracted from sludge and domestic animal feedlot soils, all with high organic carbon contents. After DNA sequencing, phylogenetic analysis identified many new *nosZ* sequences with high levels of homology to *nosZ* from Bacteroidetes, probably because of the high sequence similarity of *nosZ* from Firmicutes and Bacteroidetes, and a predominance of Bacteroidetes in feedlot environments. Three sets of new quantitative real-time PCR (qPCR) primers based on our clone library sequences were designed and tested for their specificities. Our data showed that only Bacteroidetes-related *nosZ* sequences were amplified, whereas conventional Proteobacteria-based primers amplified only Proteobacteria-related *nosZ*. Quantitative analysis of *nosZ* with the new qPCR primers recovered ~10^4 copies per 100 ng DNA. Thus, it appears that amplification with conventional primers is insufficient for developing an understanding of the diversity and abundance of *nosZ* genes in the environment.

Received 23 April 2012
Revised 11 July 2012
Accepted 22 November 2012

INTRODUCTION

Denitrification is a series of reactions in which oxidized nitrogen is reduced to dinitrogen gas. Four microbial enzymes are required for this process, including nitrate reductase ($NO_3^- \rightarrow NO_2^-$), nitrite reductase ($NO_2^- \rightarrow NO$), nitric oxide reductase ($NO \rightarrow N_2O$) and nitrous oxide reductase ($N_2O \rightarrow N_2$). Denitrification is a route of nitrogen loss in agricultural fields (Freney *et al.*, 1990) and is involved in bioremediation of nitrate contamination (Smith *et al.*, 1994). Nitrous oxide gas produced during denitrification is a well-known greenhouse gas (Singh *et al.*, 2010) and is associated with the destruction of the ozone layer (Ravishankara *et al.*, 2009). Since this process is of biogeochemical, agricultural and environmental importance, it has been studied extensively by isolation of denitrifying bacteria (Heylen *et al.*, 2006), characterization of protein structure (Coelho *et al.*, 2011), and PCR-based detection of nitrogen cycle genes from denitrifying bacteria and environmental samples (Hallin & Lindgren, 1999).

Although many Firmicutes and Bacteroidetes strains have been identified as denitrifiers (Liu *et al.*, 2008), PCR primers have only been designed against denitrification genes from Proteobacteria; other groups of bacteria have not been considered during the efforts to reassess PCR primers for denitrification gene diversity surveys (Smith *et al.*, 1994; Scala & Kerkhof, 1998; Nogales *et al.*, 2002; Throbäck *et al.*, 2004). Thus, the sequences and diversity of denitrification genes remain poorly explored. Recently, all the genes required for denitrification were identified in the genome of *Geobacillus thermodenitrificans* NG80-2, and it was in this species that *nosZ* functionality was first proven experimentally for the Gram-positive bacteria (Feng *et al.*, 2007; Liu *et al.*, 2008).

Along with an earlier report describing primer mismatches for amplifying denitrification genes, the discovery of *nosZ* sequences from Gram-positive strains gave rise to questions about the ability of previously designed primers to detect diverse *nosZ* genes (Green *et al.*, 2010). Since

Abbreviation: qPCR, quantitative real-time PCR.

Two supplementary figures are available with the online version of this paper.

Firmicutes and *Bacillus* species represent a significant proportion of soil micro-organisms, it is clear that a thorough understanding of *nosZ* diversity and abundance will require reassessment of *nosZ* primer design (Chen *et al.*, 2012; Felske *et al.*, 1998; Guo *et al.*, 2011; Janssen, 2006). In this study, *Geobacillus*-based *nosZ* primers were designed and used to detect *nosZ* genes from 15 *Geobacillus* strains (*G. thermodenitrificans* was used a positive control), sludge, and domestic animal feeding facilities. Three quantitative real-time PCR (qPCR) primers based on clone library sequences were designed and used to quantify *nosZ* in soil environments. These results were compared with the results of amplification reactions using conventional *nosZ* primers.

METHODS

Bacterial strains and culture conditions. Fifteen *Geobacillus* species were used in this study: *G. thermodenitrificans* KACC 11363^T (DSM 465^T), *Geobacillus debilis* KACC 12204^T (DSM 16016^T), *Geobacillus thermoleovorans* KACC 11374^T (ATCC 43513^T), *Geobacillus thermocatenulatus* KACC 11364^T (DSM 730^T), *Geobacillus subterraneus* KACC 11369^T (DSM 13552^T), *Geobacillus vulcani* KACC 11367^T (DSM 13734^T), *Geobacillus pallidus* KACC 11365^T (ATCC 51176^T), *Geobacillus caldoxylosilyticus* KACC 11366^T (ATCC 700356^T), *Geobacillus jurassicus* KACC 12202^T (DSM 15726^T), *Geobacillus* sp. KACC 11425, *Geobacillus gargensis* KACC 11844^T (DSM 15378^T), *Geobacillus tepidamans* KACC 11371^T (DSM 16325^T), *Geobacillus uzenensis* KACC 11368^T (DSM 13551^T), *Geobacillus zalihae* KACC 14512^T (DSM 18318^T) and *Geobacillus zalihae* KACC 11510^T (DSM 15325^T); *G. pallidus* has been reclassified as *Aeribacillus pallidus* (Miñana-Galbis *et al.*, 2010). All the strains were purchased from the Korean Agricultural Culture Collection (KACC) and grown on nutrient agar at 55 °C. *Pseudomonas aeruginosa* PAO1 and *Escherichia coli* were grown in Luria–Bertani medium at 37 °C.

PCR amplification and primer design. Primers are listed in Table 1. PCRs were conducted at 95 °C for 90 s, followed by 35 cycles at 95 °C for 24 s, at 56 °C for 24 s, 58 °C for 24 s, and a final extension step at 72 °C for 5 min using a Mastercycler PCR machine (Eppendorf). The primer design was based on an alignment of *nosZ* genes from *G. thermodenitrificans* NG80-2 and 13 Bacteroidetes species (*Salinibacter ruber* DSM 13855, *Marivirga tractuosa* DSM 4126, *Capnocytophaga gingivalis* JCVIHMP016, *Psychroflexus torquis* ATCC 700755, *Cellulophaga algicola* DSM 14237, *Gramella forsetii* KT0803, *Robiginitalea biformata* HTCC 2501, *Maribacter* sp. HTC C2170, *Dyadobacter fermentans* DSM 18053, *Flavobacteriaceae* bacterium 3519-10, *Riemerella anatipestifer* DSM 15868, *Pedobacter saltans* DSM 12145 and *Rumella slithyformis* DSM 19594), as shown in Figs 1 and 2. Nitrous oxide reductase contains a conserved COX2 (cytochrome *c* oxidase subunit II) domain, and these primers amplify the N terminus of this domain and the flanking regions.

Primers were designated nosZGeoF and nosZGeoR (654 bp predicted product size). These primers were used to detect *nosZ* genes from *Geobacillus* species and to construct a clone library. The presence of *nosZ* in *Geobacillus* was reconfirmed using another primer set, nosZF478 and nosZR915 (437 bp amplification product). The design of nosZF478 and nosZ915 was based on the *nosZ* gene of *G. thermodenitrificans*.

Quantification of *nosZ* in the soil environment was performed via qPCR. It was not possible to develop a qPCR primer to cover all the cloned sequences due to low sequence similarities and a lack of conserved regions in the forward primer regions. Therefore, clone libraries were divided into groups based on phylogenetic clades and designated groups A, B and C (Fig. 2). Sequence alignment within each group revealed a region sufficiently conserved to enable primer design. For qPCR amplification, only three new forward primers were designed using the conserved regions found in each group (A, B and C in Fig. 2). We reused our reverse primer (nosZGeoR primer) used in clone library construction because it could bind to all three groups. Primers were chosen using Primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast) and specificity was tested by performing PCR. The reactions contained 12.5 µl of iQ SYBR Green Supermix (Bio-Rad), 0.5 µM of each primer and 50 ng soil DNA in a total volume of 25 µl. The PCR conditions were 95 °C for 3 min, followed by 40 cycles of 24 s at 95 °C, 24 s at 60 °C, and 24 s at 72 °C (iCycler iQ, Bio-Rad). Melting curve analysis supported the specificity of the qPCR primers (see Fig. S1 available with the online version of this paper). To generate a standard curve for nosZ1F-nosZ1R and nosZ2F-nosZ2R, the PCR product from *P. aeruginosa* PAO1 was cloned into the pGEM-T Easy vector system (Promega) and transformed into *E. coli* Top10. Isolated plasmid DNA was digested with *Sph*I and used as a template for amplification. Standard curves of

Table 1. Primers used in this study

Primer	Sequence (5′–3′)	Product size (bp)	Reference or source
nosZ-F	CGYTGTTCMTCGACAGCCAG	453	Kloos *et al.* (2001)
nosZ-R	CGSACCTTSTTGCCSTYGCG		
nosZGeoF	TCRTCTGAAGTYGTRAAATGG	654	This study
nosZGeoR	CTTGRTGCARCGCVGAACAGA		
nosZF478	TGTGCCGCGTTTGTGACTGAGAAT	437	This study
nosZR915	GGCGACCGGTACCAAATAAATG		
nosZ1F	WCSYTGTTCMTCGACAGCCAG	259	Henry *et al.* (2006)
nosZ1R	ATGTCGATCARCTGVKCRTTYTC		
nosZ2F	CGCRACGGCAASAAGGTSMSSGT	267	Henry *et al.* (2006)
nosZ2R	CAKRTGCAKSGCRTGGCAGAA		
nosZqPCRF1*	TCGARCAGGAYTGGRACATYCT	162	This study
nosZqPCRF2*	ACAGGAYTATGATGTRCCGCACGGA	158	This study
nosZqPCRF3*	TGACYGCCATYCGTTCWCACYTT	246	This study

*nosZGeoR was used as a reverse primer.

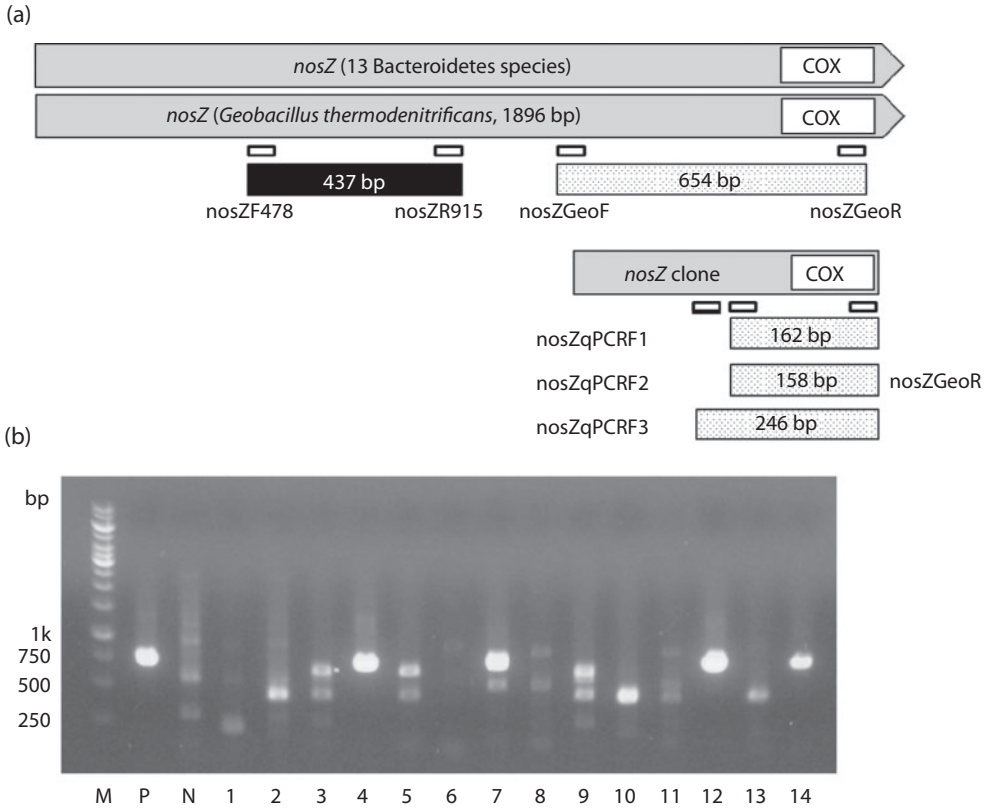

Fig. 1. Detecting *nosZ* sequences in *Geobacillus* species by using new primers. (a) Annealing positions and PCR product sizes. nosZGeoF and nosZGeoR primers were designed based on the alignment of *nosZ* genes from *G. thermodenitrificans* and 13 Bacteroidetes species. The presence of *nosZ* in *Geobacillus* species was determined using nosZGeoF/nosZGeoR and nosZF478/nosZR915. A *nosZ* clone library was constructed from soil DNA using nosZGeoF/nosZGeoR. Quantitative analysis of *nosZ* in soil DNA was performed using nosZqPCR1–3 (forward) and nosZGeoR (reverse). PCR products used in the clone library and qPCR analysis contained the cytochrome *c* conserved domain (COX). (b) PCR amplification of *nosZ* from *Geobacillus* species using *nosZ* primers designed against *G. thermodenitrificans* NG80-2 *nosZ*. PCR products of the expected size were amplified from *G. thermodenitrificans* as a positive control and four other *Geobacillus* species. M, 1 kb DNA ladder; P, positive control, *G. thermodenitrificans* KACC 11363^T (DSM 465^T); N, negative control, *P. aeruginosa* PAO1; 1, *G. debilis* KACC 12204^T (DSM 16016^T); 2, *G. thermoleovorans* KACC 11374^T (ATCC 43513^T); 3, *G. thermocatenulatus* KACC 11364^T (DSM 730^T); 4, *G. subterraneus* KACC 11369^T (DSM 13552^T); 5, *G. vulcani* KACC 11367^T (DSM 13734^T); 6, *G. pallidus* KACC 11365^T (ATCC 51176^T); 7, *G. caldoxylosilyticus* KACC 11366^T (ATCC 700356^T); 8, *G. jurassicus* KACC 11202^T (DSM 15726^T); 9, *Geobacillus* sp. KACC 12202; 10, *G. gargensis* KACC 13844^T (DSM 15387^T); 11, *G. tepidamans* KACC 11371^T (DSM 16325^T); 12, *G. uzenensis* KACC 11368^T (DSM 13551^T); 13, *G. zalihae* KACC 14512^T (DSM 18318^T); and 14, *G. lituanicus* KACC 11510^T (DSM 15325^T). Currently, *G. pallidus* is reclassified as *Aeribacillus pallidus*.

nosZqPCRF1 (group A), nosZqPCRF2 (group B) and nosZqPCRF3 (group C) with nosZGeoR were generated using linearized plasmids isolated from clone libraries UR11, WD07 and SL01, respectively. The following are the equations for the standard curves for the qPCR primers used in this study: nosZ1F and nosZ1R, $y=-1.503\ln(x)+41.456$ ($R^2=0.9923$); nosZ2F and nosZ2R, $y=-1.623\ln(x)+40.497$ ($R^2=0.9925$), nosZqPCRF1 and nosZGeoR, $y=-2.00\ln(x)+46.28$ ($R^2=0.9949$); nosZqPCRF2 and nosZGeoR, $y=-1.51\ln(x)+35.86$ ($R^2=0.9802$); and nosZqPCRF3 and nosZGeoR, $y=-1.58\ln(x)+36.81$ ($R^2=0.9925$; $y=C_t$, x=copy number per microlitre). We attempted to design a qPCR primer (nosZqPCRF3/ nosZGeoR pair) for group C with a product size of less than 200 bp; however, the primers failed to amplify a clear PCR band. The mean copy number and standard deviations were calculated from experiments performed in triplicate.

Soil samples and DNA extraction. Twelve environmental samples were collected from different sources, including one sludge sample from a wastewater treatment plant (Jungnang water-recycling centre, Seoul, Korea), one sample from playground soil (Korea University, Seoul, Korea), one sample from an abandoned mine soil (Gahak mine, Gwangmyeong, Korea), five soil samples from domestic animal feeding facilities (designated WD, UR, HD, YM and SJ, all located in Gyeonggi province of Korea), three samples from Antarctic soil (King Sejong Station, 62° 13′ 25.1S, 58° 47′ 10.4E; Cape Burk 1, 74° 45′ 32.6N, 136° 48′ 743E; and Cape Burk 2, 74° 45′ 99.9N, 136° 47′ 974E), and one sample from forest soil (37° 37′ 13N, 126° 58′ 54E). The samples were stored in a freezer (<−80 °C) prior to DNA extraction. Soil DNA was extracted from 250 mg of soil using a NucleoSpin Soil kit (Machery-Nagel) according to the manufacturer's instructions. Soil DNA concentrations were measured using a

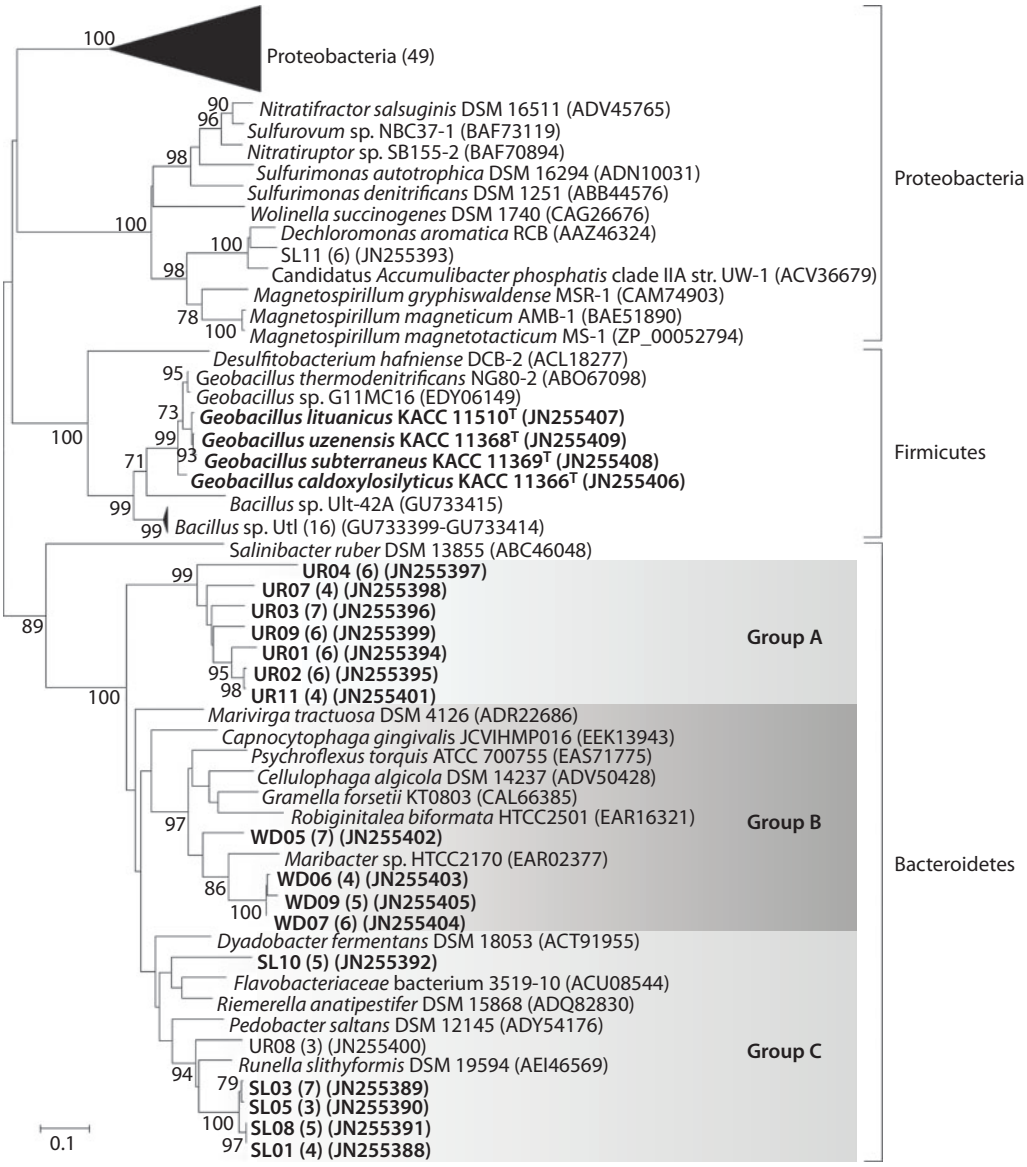

Fig. 2. Phylogenetic analysis of *nosZ* clone libraries constructed with PCR products amplified by nosZGeoF and nosZGeoR primers. After alignment and trimming of unaligned sequence, 200 amino acids (600 bp) were used for phylogenetic analysis. A neighbour-joining phylogenetic tree is shown of NosZ sequences available from GenBank, including Proteobacteria, Firmicutes, Bacteroidetes and translated sequences of the clone libraries. Black triangles indicate a collapsed subtree of the monophyletic Proteobacteria group, and the number of sequences in a subtree is in parentheses. Most clone library sequences were associated with Bacteroidetes NosZ. Bootstrap values above 70 % are labelled at the nodes (n=1000). Sequences from this study are shown in bold type. The numbers of clone library sequences with the same RFLP pattern are shown in parentheses. Scale bar, 0.1 amino acid change per sequence position.

NanoDrop Spectrophotometer ND-1000 (Thermo Fisher Scientific). PCR amplifications were performed under the same conditions described for the bacterial strains. The absence of PCR inhibitor was tested according to Nõlvak *et al.* (2012). The PCR recovery rate was tested using pGEM-nosZ$_{PAO1}$ or a mixture of 1 µl soil DNA and a known concentration of pGEM-nosZ$_{PAO1}$. T7 and nosZ1R primers were used to amplify the PCR product only from pGEM-nosZ$_{PAO1}$.

***nosZ* library construction.** PCR products amplified by nosZGeoF and nosZGeoR from the soil DNA of four environmental samples were cloned into pGEM-T Easy, and 100 clone libraries (25 from each sample) were constructed. RFLP analyses were conducted on PCR products digested with *Hae*III. RFLP patterns were observed after 0.8 % agarose gel electrophoresis (100 V, 30 min). Eighteen clones that exhibited differing RFLP patterns were chosen for sequencing with the T7 primer (5′-TAATACGACTCACTATAGGG-3′). Nucleotide sequences were translated by using the Translate tool (http://web.expasy.org/translate/), and NosZ sequences from the GenBank database were aligned and analysed with MEGA 5.05 software using CLUSTAL W and the neighbour-joining algorithm. For

phylogenetic analysis, 200 amino acid sequences were used after alignment and trimming of unaligned sequences from 218 amino acids translated from 654 bp. Sequences from WD01 and WD04 were omitted because of internal stop codons and the absence of BLAST matches, respectively. To confirm the fidelity of the new *nosZ* qPCR primers, additional clone libraries were constructed using the three qPCR primer pair sets and conventional *nosZ* qPCR primers, nosZ1F, nosZ1R, nosZ2F and nosZ2R, for comparison (Table 1) (Henry *et al.*, 2006). All the procedures and conditions for PCR, cloning, RFLP and sequencing were identical to those for the nosZGeoF/nosZGeoR libraries except the restriction enzyme used, which was *Msp*I. All the sequencing results were checked manually to ensure high quality. The clone libraries of *nosZ* nucleotide sequences from *Geobacillus* species are shown in Fig. 2 (GenBank accession nos JN255388–JN 255409). Nucleotide sequences of qPCR products were not deposited, because GenBank does not accept nucleotide sequences less than 200 bp.

RESULTS AND DISCUSSION

Primer mismatches

To investigate the specificity of previously published conventional *nosZ* primers for Gram-positive *nosZ* genes, 13 primers were chosen from six references (Chèneby *et al.*, 1998; Scala & Kerkhof, 1998; Kloos *et al.*, 2001; Nogales *et al.*, 2002; Throbäck *et al.*, 2004; Pujol Pereira *et al.*, 2011). These primers were aligned with the *nosZ* sequence from *G. thermodenitrificans* NG80-2 by using MEGA 5.05. A significant number of mismatches were detected, as the annealing sequences were different in the Gram-positive bacteria. The number and percentage (mismatches/total length of primer regardless of degeneracy) of mismatches for nos661F, nosZ661b, nosLb, nos1527F, nosZ-F, nos1527R, nos1773R, nosZ1773b, nosRb, nosZ1622R, nosZ-R, nosZ2F and nosZ2R were 9 (50 %), 7 (39 %), 8 (38 %), 10 (53 %), 6 (30 %), 6 (33 %), 4 (20 %), 7 (21 %), 7 (33 %), 8 (40 %), 10 (48 %), 9 (39 %) and 7 (33 %), respectively. If any nucleotide of degenerate code was present, it was considered as matching sequences. Given primer lengths of 18–21 bp, these mismatches precluded specific amplification of *nosZ* from *G. thermodenitrificans* NG80-2.

Detection of *nosZ* from *Geobacillus* species

Primer mismatches observed in *in silico* analysis prompted us to experimentally confirm whether conventional primers could amplify the *nosZ* gene from non-Proteobacteria species. Primer specificity testing was performed with a conventional primer set, nosZ-F and nosZ-R (Kloos *et al.*, 2001). This primer set was chosen because it had been used by many researchers to study denitrifying bacteria in diverse environments such as soils, activated sludge and wetlands (Jung *et al.*, 2011). Amplification with nosZ-F and nosZ-R was unsuccessful for 15 of the tested *Geobacillus* species. Although amplification reactions using *G. thermocatenulatus*, *G. subterraneus* and *G. pallidus* templates generated PCR products of the expected size, many non-specific bands were also observed (data not shown). The lack of amplicons from *G. thermodenitrificans* indicates that conventional *nosZ* primers are inappropriate for detecting *nosZ* genes from a particular group of bacteria such as the *Geobacillus* species.

Detection of *nosZ* with a newly designed *Geobacillus*-based primer pair (nosZGeoF and nosZGeoR) was performed. Among the 15 *Geobacillus* species examined, products of the expected size were amplified from *G. subterraneus*, *G. caldoxylosilyticus*, *G. uzenensis* and *G. lituanicus* (Fig. 1b). The presence of the *nosZ* gene in these *Geobacillus* species was reconfirmed by using a second primer set (nosZF478, nosZR915), which generated results consistent with those obtained for nosZGeo primers (data not shown). Amino acid sequences of the four *Geobacillus* species (*G. subterraneus*, *G. caldoxylosilyticus*, *G. uzenensis* and *G. lituanicus*) exhibited 94.0–96.5 % sequence similarity to *G. thermodenitrificans* NG80-2 NosZ, suggesting that these sequences are closely related (Fig. 2). Over the course of the study, genomes of several *Geobacillus* species were sequenced, and currently a total of 14 genomes are available and 14 additional *Geobacillus* strains are registered to the NCBI Bioproject for genome sequencing. A search of NosZ according to the predicted function and BLASTP demonstrated the absence of NosZ from *Geobacillus kaustophilus* HTA426, *G. thermoleovorans* CCB_US3_UF5, *Geobacillus thermoglucosidasius* C56-YS93, *Geobacillus thermoglucosidans* TNO-09.020 and five unnamed *Geobacillus* strains, which is consistent with our data (Figs 1 and 2). *P. aeruginosa* PAO1 and several *Flavobacterium* species are also known to contain *nosZ* genes; however, they were not detected by the *nosZ* primers designed in this study (data not shown).

Diversity of *nosZ* in soils

PCR amplification with nosZGeoF and nosZGeoR primers was performed on 12 soil samples, including a sludge from a wastewater treatment plant, a playground soil, an abandoned mine soil, five domestic animal feeding facilities soil, three Antarctic soils and a forest soil. *nosZ* PCR products of the expected size were amplified from only four samples: a sludge sample (SL) and three domestic animal feedlot soils (designated SJ, UR and WD; data not shown). Clone libraries were constructed using the PCR product of nosZGeoF and nosZGeoR primers from the SJ, SL, UR and WD samples. Most clone library sequences were associated with nitrous oxide reductases from the Bacteroidetes, probably because all the Bacteroidetes species shown in Fig. 2 share nucleotide sequences to which nosZGeoF and nosZGeoR primers can anneal. One of the clone libraries, SL11, contained the *nosZ* sequence, which is more closely related to Proteobacteria *nosZ* genes. We assumed that the *nosZ* gene of SL11 differs from that of *P. aeruginosa*, which we used as a negative control in the Fig. 1. Our data also suggested that a previously known conventional Proteobacteria-based *nosZ* primer could not cover all Proteobacteria *nosZ* genes. The predominance of Bacteroidetes in animal faeces may have enriched the soil

samples with Bacteroidetes and Firmicutes (Durso *et al.*, 2011). The sludge and feedlot soils which produced a PCR product amplified by nosZGeoF and nosZGeoR primers have higher total organic carbon [TOC; (24253.6–50260.4 mg kg^{-1})] than other soils (416.2–5641.1 mg kg^{-1}). Therefore, the *nosZ* primers designed in this study were able to amplify the *nosZ* sequence from Firmicutes and Bacteroidetes.

nosZ PCR detection with conventional *nosZ* qPCR primers

On the basis of the phylogenetic analysis of *Geobacillus* species' *nosZ* and clone libraries from environmental samples, it appears that the use of conventional primers may have resulted in a significant underestimation of environmental *nosZ* diversity (Henry *et al.*, 2006). To confirm that diverse *nosZ* sequences were detected in qPCR analysis with conventional qPCR primers, we constructed two clone libraries using nosZ1F-nosZ1R and nosZ2F-nosZ2R primers targeting different regions of *nosZ* sequences. Sequencing and phylogenetic analysis of clone libraries demonstrated that only Proteobacteria *nosZ* sequences were amplified (Fig. S2), even though the presence of Bacteriodetes-associated *nosZ* was confirmed via the nosZGeoF-nosZGeoR primer clone library (Fig. 2). These results strongly suggest that a certain portion of *nosZ* gene abundance may have been neglected in gene abundance and diversity surveys performed with conventional primers.

Primer specificity of a new Bacteroidetes-based qPCR primer

Recognizing the inadequacy of conventional *nosZ* primers, we sought to design new qPCR primers to detect Bacteroidetes-associated *nosZ* sequences. A qPCR primer covering all Bacteroidetes-associated *nosZ* was not feasible because *nosZ* sequences are too divergent and there are no conserved nucleotide sequences for primer annealing. Based on phylogenetic analysis, we divided the *nosZ* sequences into the groups designated A, B and C, as shown in Fig. 2. Alignment of *nosZ* sequences within each group revealed highly conserved nucleotide sequences for primer annealing. Three forward primers, nosZqPCRF1, nosZqPCRF2 and nosZqPCRF3, were designed, and nosZGeoR was used as a reverse primer (Fig. 1). *nosZ* of

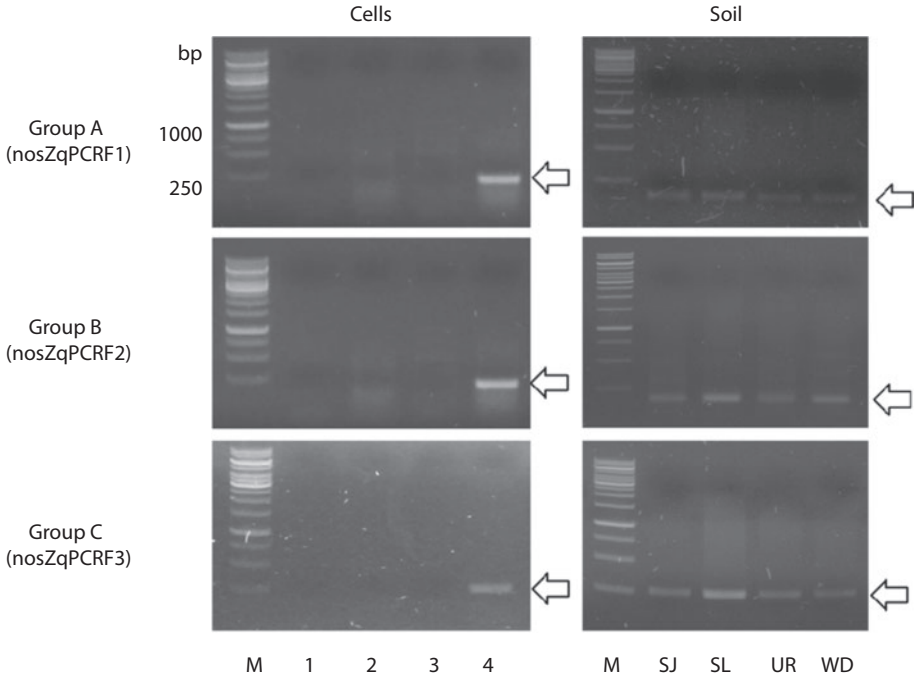

Fig. 3. Specificity tests of the qPCR primers. nosZqPCRF1, nosZqPCRF2 and nosZqPCRF3 were designed to specifically amplify *nosZ* genes affiliated with groups A, B and C, as shown in Fig. 2. For specificity tests with cells, (lane 1) *E. coli* Top10 (pGEM-T easy) (host cell of *nosZ* clone library), (lane 2) *P. aeruginosa* PAO1 (*nosZ*-containing Proteobacteria) and (lane 3) *G. thermodenitrificans* (*nosZ*-containing Firmicutes) were used as negative controls. Lane 4: clone libraries UR11, WD07 and SL01 were positive controls for nosZqPCRF1, nosZqPCRF2 and nosZqPCRF3, representing groups A, B and C, respectively. For specificity tests with soil DNA, four soil samples were used: SJ, SL, UR and WD. Arrows indicate the expected PCR product size. All the PCR results show a PCR product of the desired size, indicating that the new qPCR primers specifically amplify *nosZ* genes from cells and soil DNA. M, 1 kb ladder.

G. thermodenitrificans could not be used for designing our qPCR forward primer because a short DNA region must be used for qPCR and the *nosZ* forward primer region of *G. thermodenitrificans* differs from those of groups A, B and C shown in Fig. 2. An *in silico* primer specificity check using primer-BLAST predicted that nosZqPCRF2 and nosZqPCR3 could bind to the nitrous oxide reductase genes of *D. fermentans* DSM 18053 and *Haliscomenobacter hydrossis* DSM 1100, respectively. However, there was no sequence at which the nosZqPCRF1 and nosZGeoR primers could amplify in GenBank. This may reflect the absence of closely related *nosZ* sequences within group A in GenBank, as shown in Fig. 2. To test qPCR primer specificity, non-*nosZ*-containing strain *E. coli*, *nosZ*-containing Gram-negative strain *P. aeruginosa* PAO1, and *nosZ*-containing Gram-positive strain *G. thermodenitrificans* were used as negative controls. As shown in Fig. 3, the new qPCR primers successfully amplified *nosZ* from representatives of clone libraries (group A, UR11; group B, WD07; group C, SL01). PCR with soil DNA using the new qPCR primers also generated PCR products of the desired length (Fig. 3). These specificity tests lend support to the suitability of the new qPCR primers for quantitative analysis of *nosZ* associated with Bacteroidetes.

nosZ PCR detection with new *Bacteroidetes*-based *nosZ* qPCR primers

To confirm that our new qPCR primers actually amplified *nosZ* genes from soil DNA, clone libraries were constructed with nosZqPCR1, nosZqPCR2 and nosZqPCR3. Fifty-eight clones were constructed from qPCR products and were subjected to RFLP analysis. Two clones could not be analysed in the phylogenetic tree because they had no database match after sequencing. After DNA sequencing of the clone with the different RFLP patterns, phylogenetic analysis of the amino acid sequences of the qPCR clone libraries showed that most qPCR products were associated with previously known Bacteroidetes *nosZ* genes (Fig. 4). Phylogenetic analyses revealed that qPCR products amplified by different sets of primers were not distinguishable from each other. Because only a short sequence fragment (50 amino acids after

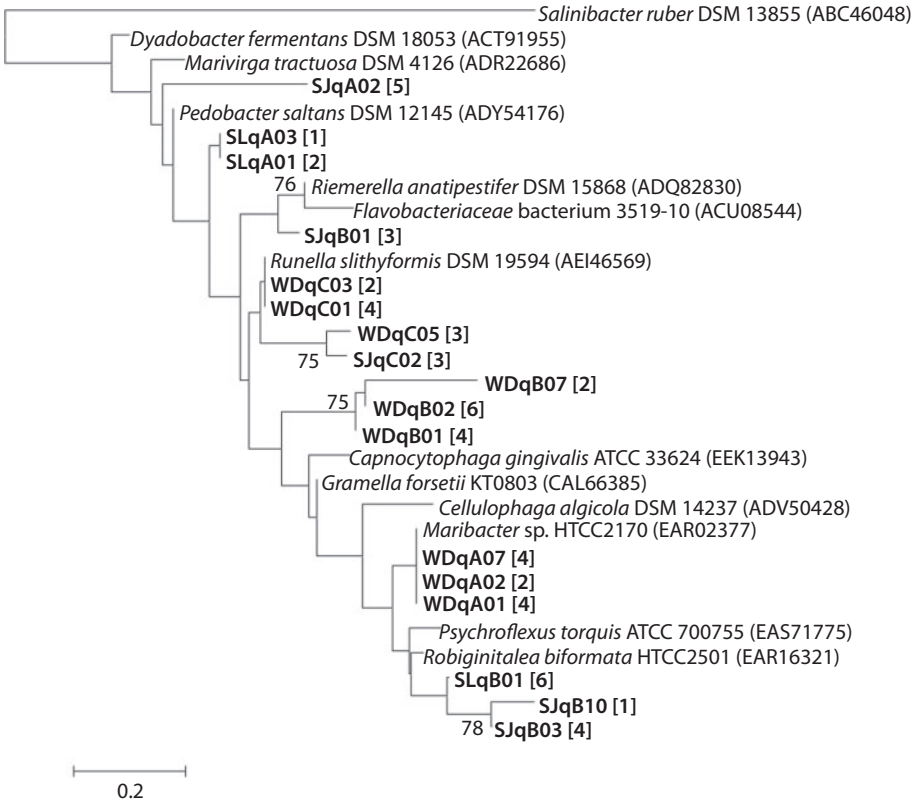

Fig. 4. Neighbour-joining phylogenetic tree of *nosZ* amplified by qPCR. To confirm that the new qPCR primers amplified *nosZ* genes from soil DNA, clone libraries were prepared with the qPCR products shown in bold type. Clone names contained six letters indicating the sources of DNA (SJ, SL, UR, WD), PCR products (q, qPCR), PCR primer (A, nosZqPCRF1; B, nosZqPCRF2; C, nosZqPCRF3), and a two-digit serial number. The number of identical RFLP patterns and GenBank accession number are shown in brackets and parentheses, respectively. After alignment and trimming of unaligned sequence, 50 amino acids (150 bp) were used for phylogenetic analysis. *S. ruber* DSM 13855 was used as an outgroup. Bootstrap values greater than 70 are shown at nodes. Scale bar, 0.2 substitutions per amino acid sequence.

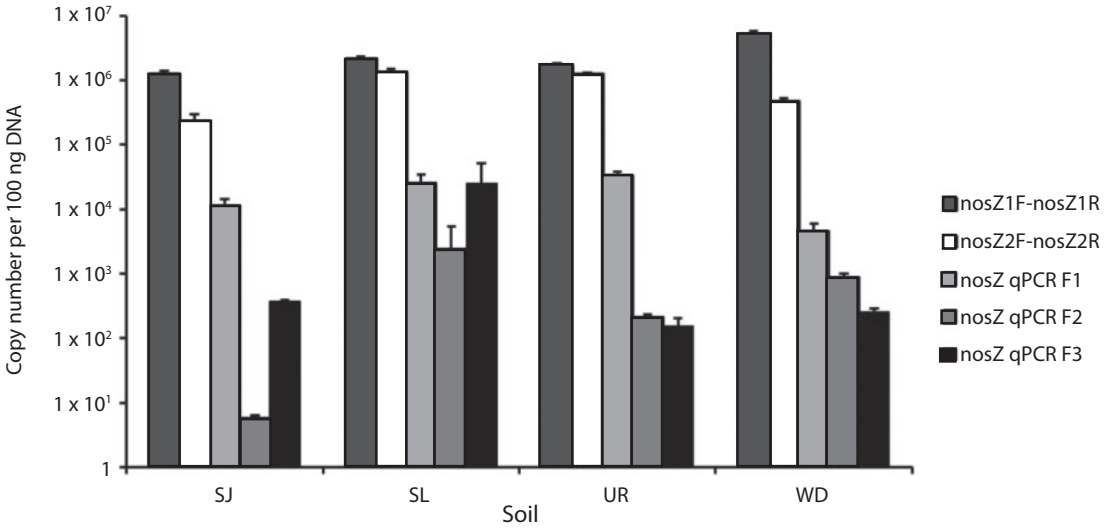

Fig. 5. Quantification of *nosZ* from diverse environments using the conventional (nosZ1F-nosZ1R and nosZ2F-nosZ2R) and new qPCR primers (forward primers nosZqPCRF1, nosZqPCRF2 and nosZqPCRF3, corresponding to groups A, B and C, respectively; nosZGeoR alone was used as a reverse primer) designed in this study. Primers were intended to amplify *nosZ* associated with Bacteroidetes based on the clone library sequences (see Fig. 2). When the new primers were utilized, 2–5 % of the copy number was obtained compared with the results of conventional qPCR primers. Means and standard deviations are shown for triplicate experiments. SL, sludge sample; SJ, UR and WD, soil samples.

translation and alignment) of the qPCR product was used for the phylogenetic analysis shown in Fig. 4, the phylogenetic information used in grouping in Fig. 2 was not used.

Quantification of *nosZ* in soils with new Bacteroidetes-based primers

To compare the amplification characteristics of five qPCR primers (two conventional primers: nosZ1F-nosZ1R and nosZ2F-nosZ2R; three new primers: nosZqPCR1F, nosZqPCR2F and nosZqPCR as forward primers, and nosZGeoR alone as a reverse primer) qPCR analyses were performed on the same soil DNA (four samples: SJ, SL, UR and WD) used for *nosZ* library construction shown in Fig. 2. As shown in Fig. 5, the conventional qPCR *nosZ* primers (Henry *et al.*, 2006) nosZ1F-nosZ1R and nosZ2F-nosZ2R amplified substantial amounts of *nosZ* from the environmental samples SL, SJ, UR and WD (nosZ1F-nosZ1R: $1.27 \pm 0.12 \times 10^6$, $2.16 \pm 0.20 \times 10^6$, $1.69 \pm 0.13 \times 10^6$ and $5.24 \pm 0.54 \times 10^5$ copies per 100 ng DNA, respectively; nosZ2F-nosZ2R: $2.33 \pm 0.60 \times 10^5$, $1.33 \pm 0.17 \times 10^6$, $1.25 \pm 0.05 \times 10^6$ and $4.68 \pm 0.61 \times 10^5$ copies per 100 ng DNA, respectively).

Quantification of *nosZ* with the new qPCR primers revealed a significant amount of Bacteroidetes *nosZ* genes in soils (Fig. 5). Group A *nosZ* sequences were the most abundant in all the four soil DNAs [1.13×10^4, 2.58×10^4, 3.29×10^4 and 4.55×10^3 copies (g dry soil)$^{-1}$ in SJ, SL, UR and WD, respectively]. Copy numbers of the other types of *nosZ* sequences were relatively low in samples SJ [5.81 and 3.68×10^2 copies (g dry soil)$^{-1}$, groups B and C]. SL (sludge sample) contained the greatest amount of *nosZ* genes [2.43×10^3 and 2.52×10^4 copies (g dry soil)$^{-1}$, groups B and C]. When the new primers were utilized, 2–5 % of the copy number was obtained compared with the results for the conventional qPCR primers nosZ1F-nosZ1R and nosZ2F-nosZ2R.

In this study, new qPCR primers targeting Bacteroidetes were designed and proven valuable for *nosZ* detection and quantification. In a related study, using *nosZ* primers designed against *Desulfitobacterium hafniense* Y-51 and *G. thermodenitrificans* NG80-2, Jones *et al.* (2011) identified *nosZ* genes from 14 nitrogen- and nitrous oxide-producing *Bacillus* soil isolates. Those primers may be useful for amplification of closely related *nosZ* sequences; however, the large PCR product size (1502 bp) may have impeded the quantitative analyses. Nitrous oxide reductase is a good genetic marker for nitrous oxide reduction, since its function is specific to that process, while other denitrification reductases participate in additional pathways (Zumft, 1997; Suharti & de Vries, 2005; Basaglia *et al.*, 2007; Zumft & Bothe, 2007). Therefore, improvements to the *nosZ* primer design will continue to assist in developing a better understanding of the abundance and diversity of genes underlying the denitrification process.

ACKNOWLEDGEMENTS

This study was supported by a grant from the Polar Academy Program (PAP) of the Korean Polar Research Institute (KOPRI).

W. P.'s work was supported by the LG Yonam Foundation, Seoul, South Korea. This work was also supported by the National Institute of Environmental Health Sciences of the National Institutes of Health under award number P42ES004699. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

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Edited by: E. L. Madsen