

Analysis of denitrification genes and comparison of *nosZ*, *cnorB* and 16S rDNA from culturable denitrifying bacteria in potato cropping systems[☆]

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Received 8 May 2006

Abstract

Bacterial denitrification in agricultural soils is a major source of nitrous oxide, a potent greenhouse gas. This study examined the culturable bacterial population of denitrifiers in arable field soils in potato (*Solanum tuberosum* L.) production and denitrification genes (*nir*, *nor* and *nos*) and 16S rDNA in those isolates. Enrichments for culturable denitrifiers yielded 31 diverse isolates that were then analysed for denitrification genes. The nitrous oxide reductase (*nosZ*) gene was found in all isolates. The majority of isolates (~90%) contained the *cnorB* nitric oxide reductase gene, with the remainder containing the *qnorB* gene. Nitrite reductase genes (*nirS* and *nirK*) were amplifiable from most of the isolates, and were segregated between species similar to previously isolated denitrifiers. Isolated strains were preliminarily identified using fatty acid methyl ester analysis and further identified using 16S rDNA sequencing. The majority of isolates (21) were classified as *Pseudomonas* sp., with smaller groups of isolates being most similar to *Bosea* spp. (4), *Achromobacter* spp. (4) and two isolates closely related to *Sinorhizobium/Ensifer* spp. Phylogenetic trees were compared among *nosZ*, *cnorB* and 16S rDNA genes for a subset of *Pseudomonas* strains. The trees were mostly congruent, but some *Pseudomonas* sp. isolates grouped differently depending on the gene analysed, indicating potential horizontal gene transfer of denitrification genes. Although *Bosea* spp. are known denitrifiers, to the best of our knowledge this is the first report of isolation and sequencing of denitrification genes from this bacterial genus. Crown Copyright © 2006 Published by Elsevier GmbH. All rights reserved.

Keywords: Denitrification; 16S rDNA; Nitrous oxide reductase gene; Nitric oxide reductase gene; Soil; Phylogenetic analysis

Introduction

Denitrification is a process whereby alternative electron acceptors including nitrate (NO_3^-), nitrite

(NO_2^-), nitric oxide (NO) and nitrous oxide (N_2O) allow microorganisms to respire under anaerobic or oxygen-limited conditions. Nitrate and nitrite are reduced to gaseous compounds (NO, N_2O and nitrogen gas (N_2)) which are released to the atmosphere, leading to loss of plant available N from soils [41]. Incomplete denitrification can lead to emission of nitrous oxide, a potent greenhouse gas implicated in global warming and destruction of the ozone layer [10]. Denitrification from agricultural soils is a major process contributing to the

[☆]Note: Nucleotide sequences obtained in this study are available in Genbank under accession numbers DQ377742–DQ377772 (16S rDNA), DQ377773–DQ377803 (*nosZ*), DQ420236–DQ420253 (*cnorB*), DQ518185–DQ518197 (*nirS*) and DQ518198–DQ518208 (*nirK*).

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emission of nitrous oxide to the atmosphere. Agricultural soil management accounted for 67% of US nitrous oxide emissions in 2003 [36]. Increased applications of fertilizer N is believed to have led to increased nitrous oxide emissions to the atmosphere [33]. Mineral N applications, and organic amendments, generally increase total denitrification and N₂O emissions from soils [26].

Microbial community structure has a significant influence on the turnover of solutes in soil and water and on global trace gas emissions [19]. Microbial community structure is also influenced by fertilizer application regimes, and recent work has shown the effects of long-term fertilisation on denitrifier community composition [15]. Arable cropping systems, such as potato, have been shown to have relatively high emissions of nitrous oxide [29]. To the best of our knowledge, bacterial denitrifier populations and activity have not previously been assessed in relation to potato production practices and fertilizer application.

Denitrifying bacteria have been isolated from diverse environments (agricultural soils, deep sea sediments, waste-water treatment plants) and belong to diverse bacterial genera [41]. PCR primer pairs have been developed for all the functional genes in the denitrification pathway, allowing researchers an unprecedented ability to amplify and analyse these genes in cultured isolates and environmental samples [5,6,28]. Genetic analysis of denitrification usually proceeds from the nitrite reductase step (*nirK* or *nirS*), because nitrate reduction is not specific to denitrification [17]. Nitric oxide reductase genes (*cnorB* and *qnorB*) and nitrous oxide reductase gene (*nosZ*) are also included. Bacterial genome sequencing is increasing the number of predicted denitrification genes in DNA databases. Sequences of denitrification gene fragments from environmental cloning experiments have also been added to DNA databases in recent years, but phylogenetic information is usually not available to classify these sequences and compare to characterised isolates [17].

Analysis of functional genes is useful for the denitrifying population because they are more specific to the process than a phylogenetic approach. The combination of phylogenetic and functional gene information may lead to better understanding of the denitrifier population, although there is evidence that phylogenetic and functional genes may have limited correlation [14,17]. The incorporation of phylogenetic information from cultured isolates can improve the affiliation of uncultured sequences obtained from environmental cloning experiments [17]. Sequencing denitrification genes from culturable isolates also provides information on the variation in expressed functional gene sequences, which is not ensured when using environmental clones. Ultimately, analysis of

functional gene sequence expression is required to determine activity of denitrifying populations in situ [31].

The purpose of this study was to generate information about the denitrifier population in representative arable soils utilised for potato production in New Brunswick, Canada under humid, temperate climatic conditions. PCR and sequencing were used to characterise the 16S rDNA and functional denitrification genes (*nirK*, *nirS*, *cnorB*, *qnorB* and *nosZ*) in a set of enriched denitrifier isolates. Phylogenies were compared for *nosZ* and 16S rDNA gene sequences. Culturable denitrifiers were used in this study to improve our knowledge linking phylogenetic affiliation with functional genes in the denitrification process.

Materials and methods

Microorganisms and growth conditions

Control microorganisms, the denitrifier *Paracoccus denitrificans* ATCC 19367 (positive control) and the non-denitrifier *Escherichia coli* ATCC 29425 (negative control) were maintained on nutrient agar (Difco, BD-Canada, Oakville, ON) and routinely cultured at 30 or 37 °C, respectively. Soil-isolated denitrifiers were maintained on nutrient agar containing 5 mM KNO₃ and cultured at 30 °C. All strains were maintained for long-term storage at –80 °C in 15% (v/v) sterile glycerol.

Isolation and identification of denitrifiers

Enrichments for denitrifying microorganisms were performed using standard denitrifier MPN tubes [35]. Fresh soil (10 g) in 95 ml sterile phosphate-buffered saline (PBS) was blended in a Waring blender with a Pulveriser attachment (3 × 1 min, setting 5). Soil suspensions were diluted 10-fold in PBS to 10⁻⁷. Sterilised Hungate tubes containing nutrient broth (NB; Difco) plus 5 mM KNO₃ and inverted glass Durham tubes were inoculated by injection with 1 ml of each dilution from 10⁻³ to 10⁻⁷ in duplicate. Tubes were incubated at 30 °C for up to 2 weeks before testing for nitrate removal and scoring for growth and gas production. Nitrate removal was tested using Quantofix nitrate–nitrite test sticks as recommended by the manufacturer (Macherey-Nagel Inc., Easton, PA, USA). Tubes that fulfilled all three criteria (growth, nitrate removal, gas production) were considered presumptively positive for denitrification. Bacteria from positive tubes were diluted in sterile PBS and spread plated onto nutrient agar plates prior to incubation at 30 °C for 2–3 days. Individual colony types were picked from spread plates, streaked for purity three times and then presumptive denitrification

was retested in MPN tubes as described above, in duplicate. Growth, gas production and nitrate removal were again scored to confirm denitrification. The acetylene blockage method was used to ensure that the gas bubble observed in the Durham tube was due to the denitrification process [35]. MPN tubes were inoculated as above with isolated strains, except that 1 ml acetylene was injected into tubes prior to inoculation. N_2O was measured after four days incubation by GC analysis of headspace gas. Gas analysis was performed using a Varian Star 3800 Gas Chromatograph (Varian, Walnut Creek, CA) fitted with an electron capture detector. The ECD was operated at 300 °C, 10% Ar, 90% CH_4 carrier gas at 10 ml min⁻¹, Haysep N 80/100 pre-column (0.32 cm diameter × 50 cm length) and Haysep D 80/100 mesh analytical columns (0.32 cm diameter × 200 cm length) in a column oven operated at 70 °C. Pre-column was used in combination with a valve to remove water from the sample. A sample was scored as positive when the N_2O concentration in the headspace following four days incubation was greater than 200 µl l⁻¹.

A subset of positive denitrifiers with visibly differing morphologies were cultured on nutrient agar plates and analysed at the Laboratory Services Division (University of Guelph, Guelph, ON, Canada) for identification by fatty acid-based Microbial Identification SystemTM analysis [30].

Sampling site and soil characteristics

Soil was sampled on 2 November 2004, from three fields at the Potato Research Centre, Fredericton, NB, Canada (Latitude: 45° 21'N, Longitude: 66° 31'W) during typical cool, wet, late fall conditions. All fields were cropped to potato in 2004 and were in potato–cereal rotations. Soil sample characteristics were as follows: (1) pH 5.2, 19 g kg⁻¹ organic carbon, 89:606:305 clay:sand:silt g kg⁻¹, (2) pH 5.7, 17 g kg⁻¹ organic carbon, 96:507:397 clay:sand:silt g kg⁻¹ and 3) pH 5.9, 20 g kg⁻¹ and 80:675:245 clay:sand:silt g kg⁻¹. Soil pH was measured using a 1:1 soil:water extract. Soil texture was determined by the pipette method and organic carbon concentration was determined by combustion. Soil samples were taken from the A horizon (0–15 cm depth) and the B horizon (20–30 cm depth). Soil was stored in ventilated plastic buckets at 4 °C in the dark and used as soon as possible post-sampling.

PCR amplification and sequencing of denitrification and 16S rDNA genes

PCR was used to amplify denitrification genes encoding nitrite reductase (*nirK* and *nirS*), nitric oxide reductase (*cnorB* and *qnorB*) and nitrous oxide reductase

(*nosZ*) from isolated denitrifier strains. Genomic DNA was prepared from all isolates and control denitrifier strains using UltraCleanTM Microbial DNA Kit (Molecular Bio Laboratories Inc., Carlsbad, CA, USA). Genomic DNA concentrations were measured using Picogreen DNA quantitation kit (Invitrogen Canada Inc., Burlington, ON, Canada). All genomic DNA templates were diluted to ~2.5 ng µl⁻¹ and used in PCR reactions at 5 µl per 25 or 50 µl reaction. Primers for *nirS* amplification were *nirS1F* and *nirS6R* [5]. The annealing temperature used for these reactions was 60 °C, instead of the previously recommended 45–40 °C touchdown PCR [5]. *nirK* was amplified using primers CD*nirKF* (5'-TCATGGTGCTGCCGCGBGACGG-3', modification of Copper 583F; [24]) and *nirK1040* [18]. The annealing temperature used for these reactions was 52 °C. *cnorB* and *qnorB* products were amplified using previously described primers and conditions [6]. *nosZ* products were amplified using *nosZF-1181* and *nosZR-1880* [28]. PCR products were purified using QIAEX II Gel Extraction Kit (Qiagen Inc, Mississauga, ON, Canada), quantified and sequenced (Applied Biosystems 3730 DNA analyzer, Big Dye Terminator V3.1) with appropriate custom primers (Robarts Research, London, ON, Canada).

16S rDNA was amplified using primers 27f and 1492r [23] and conditions as described in [11]. PCR products were purified using Qiaquick PCR Purification kit (Qiagen) and sequenced as above.

Sequence analysis

Sequence chromatograms were analysed using Chromas Lite (Technelysium Pty Ltd., Tewantin, QLD, Australia). Sequences were compared with the database (Genbank) using the NCBI Blast programme [1]. Sequence editing and alignments were performed using EditSeq and MegAlign (DNASTar Inc., Madison, WI, USA). Sequences of 1386 bp (16S rDNA), 331 bp (*cnorB*) and 400 bp (*nosZ*) were used for phylogenetic analyses. Phylogenetic analysis was performed using a neighbour-joining algorithm and distance calculation [20] by using TREECON for Windows, Version 1.3b [37]. Programs from the Phylip package [16] were also used to generate maximum-likelihood trees (data not presented). For the *nosZ* alignment, the nitrous oxide reductase gene of *Azospirillum lipoferum* (AF361793) was used as outgroup. Tree topology was evaluated in all cases by bootstrap analysis (500 replicates). 16S sequences were compared with database sequences on the RDP-II website [9] to determine species affiliation of isolated denitrifiers. Chimeric 16S rDNA sequences were screened for using Pintail [3].

Results and discussion

Isolation and identification of denitrifying bacteria

MPN denitrifying tubes containing nutrient broth and nitrate were the enrichment method for denitrifying bacteria from soil samples. Denitrifiers were isolated from all three soil types and both depths sampled from each soil. Seventy-five per cent of the isolates identified were enriched from the B horizon samples (20–30 cm depth), with only 25% originating in the A horizon soil. While *Pseudomonas* spp. were found in both horizons, *Achromobacter* spp. were found primarily in the A horizon and *Bosea* spp. primarily in the B horizon. Denitrification was confirmed for all isolates using the acetylene blockage method and measurement of nitrous oxide produced (data not shown). A selection of morphologically different denitrifier isolates was chosen for preliminary identification using FAME analysis [30]. The majority of isolates were identified to be *Pseudomonas* spp., with a high similarity to *Pseudomonas fluorescens*, *Pseudomonas putida* and *Pseudomonas savastanoi* (Table 1). Other strains identified using this method included *Rhizobium radiobacter*, *Alcaligenes faecalis*, *Yersinia aldovae* and *Roseomonas fauriae*, not all of which are previously known to be denitrifiers. Due to these unusual strain identifications, further identification was carried out using 16S rRNA gene sequencing. Almost full-length sequences were obtained from all isolated strains and compared with database sequences in Genbank and RDP-II. 16S rDNA sequencing gave much higher precision to the identification of unknown isolates in this study (Table 1). Similar to the FAME ID results, the majority of strains were classified as *Pseudomonas* spp. More specifically, isolates were obtained with high similarities (94–99% 16S rDNA similarity) to newly taxonomically reclassified groups such as *Pseudomonas migulae*, *Pseudomonas mandelii*, *Pseudomonas grimontii*, *Pseudomonas lini*, *Pseudomonas kilonensis* and *Pseudomonas frederiksbergensis* [2,4,13,32,38,39]. *Pseudomonas chlororaphis* and *Pseudomonas brassicacearum* (95–98% similarity) were also identified amongst these denitrifier isolates. Nitrate reduction and denitrification can be a variable trait amongst the strains described here [13,32], but isolates of all these *Pseudomonas* strains have been previously described as positive denitrifiers [2,4,38,39]. Isolates PD 13, 17, 21 and 22 have high 16S rDNA sequence similarity to *Pseudomonas* sp. isolates or environmental clones from extreme environments such as alpine soil (AY263479; [25]), deep sea sediment (AJ551160) and soil from uranium mining waste (AM071376) (Table 1). Our sites used for soil sampling are frozen and under snow-cover for several months each year, so the similarities to *Pseudomonas* isolates from alpine environments are not unexpected.

16S rDNA sequences of strains PD 7, 19, 25 and 27 showed high similarity (94–95%) to the type strain *Achromobacter piechaudii*, but were more similar (97–98%) to an arsenite-oxidising bacterium, *Alcaligenes faecalis* (HLE) (Genbank accession no. AY027506) and *Achromobacter xylosoxidans* (AY244787). The *Achromobacter piechaudii* type strain tests negative for denitrification, but closely related strains *Achromobacter xylosoxidans* and *Achromobacter denitrificans* are known denitrifiers [8]. 16S genes of these isolates do not cluster closely with other type strains, and may comprise a novel lineage within the *Achromobacter*. Further characterisation (i.e. DNA-DNA hybridisation and phenotypic tests) is required to determine precise species affiliation.

Isolates PD 18, 19, 23 and 24 also comprise a distinct group, closely related to type strains *Bosea eneeae* (95–96%) and *Bosea vestrisii*. Highest similarity for all strains is to *Bosea vestrisii* 63286 (AF288308; [21]). *Bosea eneeae* is negative for nitrate reduction, but *Bosea vestrisii* has a variable reaction [22]. The original *Bosea* isolate, *Bosea thiooxidans*, is able to denitrify in heterotrophic growth medium containing nitrate [12]. 16S rDNA sequence is not considered a suitable marker for species delineation within *Bosea* [22], so without further classification it is not possible to say whether these isolates represent a novel lineage.

Two isolates were obtained, PD 12 and 29, similar to *Sinorhizobium fredii* (96.9%) and *Ensifer adhaerens* (98%), respectively. A denitrifying strain with 100% 16S rDNA sequence similarity to *Sinorhizobium fredii* has previously been isolated from bentgrass roots [40].

PCR of denitrification genes

Recent work in soil ecology has generated a range of primers suitable for amplifying gene fragments of denitrification genes from the environment or isolated strains [5,6,18,24,28]. PCR was used here to amplify fragments of *nirS*, *nirK*, *cnorB*, *qnorB* and *nosZ* genes, the results of which are presented in Table 2. Nitrous oxide reductase gene (*nosZ*) fragments of ~700 bp were amplified from all isolates. Although it is possible for denitrifiers to have an incomplete denitrification pathway (i.e. terminate at nitrous oxide), presence of *nosZ* in these strains was expected due to the enrichment procedure used. Use of insoluble (i.e. N₂) gas production as a positive indicator of denitrification ensures that the selected isolates proceed to the final step of the denitrification process. *nosZ* genes are highly conserved between *Pseudomonas stutzeri*, *Pseudomonas aeruginosa*, *Ralstonia eutropha*, *Pseudomonas denitrificans* and *Sinorhizobium meliloti*, as confirmed by sequencing [41]. Primers designed from these sequences are able to amplify *nosZ* from Gram-negative strains, but no Gram-positive *nosZ* gene has yet been identified. About

Table 1. Identification of denitrifier isolates from potato crop system soil samples using FAME and 16S rDNA sequencing

ID	Source	FAME	16S rDNA similarities (highest match %)	Type strain and Proteobacteria subgroup (highest match %)
PD 7	1A ^a	ND ^b	<i>Achromobacter xylosoxidans</i> ; 2002-55549; AY244787 (97.1)	<i>Achromobacter piechaudii</i> AB010841 (94.2) β
PD 20	1A	<i>Alcaligenes faecalis</i>	Arsenite-oxidizing bacterium ' <i>Alcaligenes faecalis</i> (HLE)'; AY027506 (98.2)	<i>Achromobacter piechaudii</i> AB010841 (95.0) β
PD 25	1B	<i>Alcaligenes faecalis</i>	Arsenite-oxidizing bacterium ' <i>Alcaligenes faecalis</i> (HLE)'; AY027506 (97.6)	<i>Achromobacter piechaudii</i> AB010841 (94.6) β
PD 27	3B	<i>Yersinia aldovae</i>	Arsenite-oxidizing bacterium ' <i>Alcaligenes faecalis</i> (HLE)'; AY027506 (97.5)	<i>Achromobacter piechaudii</i> AB010841 (94.5) β
PD 18	3B	<i>Roseomonas fauriae</i>	<i>Bosea vestrisii</i> ; 63286; AF288308 (96.5)	<i>Bosea eneeae</i> AF288300 (95.9) α
PD 19	3B	ND	<i>Bosea vestrisii</i> ; 63286; AF288308 (96.5)	<i>Bosea eneeae</i> AF288300 (95.9) α
PD 23	1B	<i>Roseomonas fauriae</i>	<i>Bosea vestrisii</i> ; 63286; AF288308 (96.0)	<i>Bosea eneeae</i> AF288300 (95.5) α
PD 24	1B	ND	<i>Bosea vestrisii</i> ; 63286; AF288308 (96.1)	<i>Bosea eneeae</i> AF288300 (95.5) α
PD 29	ND	ND	<i>Ensifer adhaerens</i> ; LMG 20571; AF452129 (99.2)	<i>Ensifer adhaerens</i> AY024335 (98.0) α
PD 4	2B	<i>Pseudomonas putida</i>	<i>Pseudomonas brassicacearum</i> ; 520-1; AJ292381 (100.0)	<i>Pseudomonas brassicacearum</i> AF100321 (98.5) γ
PD 5	2B	<i>Pseudomonas putida</i>	<i>Pseudomonas brassicacearum</i> ; 520-1; AJ292381 (99.6)	<i>Pseudomonas brassicacearum</i> AF100321 (98.1) γ
PD 3	2B	ND	Uncultured bacterium; BANW683; DQ264621 (97.0)	<i>Pseudomonas chlororaphis</i> AF094722 (95.7) γ
PD 14	3B	<i>Pseudomonas savastanoi</i>	Uncultured bacterium; BANW460; DQ264453 (97.0)	<i>Pseudomonas chlororaphis</i> AF094722 (95.7) γ
PD 16	3A	<i>Pseudomonas fluorescens</i>	Antarctic bacterium R-9113; AJ441004 (98.6)	<i>Pseudomonas frederiksbergensis</i> AJ249382 (94.2) γ
PD 9	3B	<i>Pseudomonas putida</i>	<i>Pseudomonas</i> sp. BWDY-24; DQ219370 (99.8)	<i>Pseudomonas grimonitii</i> AF268029 (99.2) γ
PD 10	3B	<i>Pseudomonas putida</i>	<i>Pseudomonas</i> sp. BWDY-24; DQ219370 (99.7)	<i>Pseudomonas grimonitii</i> AF268029 (99.2) γ
PD 31	2A	<i>Pseudomonas putida</i>	<i>Pseudomonas</i> sp. CM1/A2; AJ417068 (99.8)	<i>Pseudomonas kilonensis</i> AJ292426 (98.1) γ
PD 11	3B	<i>Pseudomonas fluorescens</i>	<i>Pseudomonas</i> sp. HF3/S21027; AY337597 (99.5)	<i>Pseudomonas lini</i> AY035996 (97.9) γ
PD 15	3B	ND	<i>Pseudomonas</i> sp. HF3/S21027; AY337597 (99.1)	<i>Pseudomonas lini</i> AY035996 (97.6) γ
PD 28	ND	ND	<i>Pseudomonas</i> sp. HF3/S21027; AY337597 (99.5)	<i>Pseudomonas lini</i> AY035996 (97.9) γ
PD 2	2A	<i>Pseudomonas putida</i>	<i>Pseudomonas</i> sp. BG2dii; AY263468 (98.9)	<i>Pseudomonas mandelii</i> AF058286 (98.3) γ
PD 6	2B	ND	<i>Pseudomonas</i> sp. An22; AJ551160 (96.9)	<i>Pseudomonas mandelii</i> AF058286 (96.0) γ
PD 8	1B	<i>Pseudomonas putida</i>	<i>Pseudomonas</i> sp. BG2dii; AY263468 (98.7)	<i>Pseudomonas mandelii</i> AF058286 (98.3) γ
PD 13	3B	<i>Pseudomonas putida</i>	<i>Pseudomonas</i> sp.; JG35 + U1-AG16; AM071376 (98.1)	<i>Pseudomonas mandelii</i> AF058286 (96.5) γ
PD 21	2B	<i>Pseudomonas putida</i>	<i>Pseudomonas</i> sp. An22; AJ551160 (97.7)	<i>Pseudomonas mandelii</i> AF058286 (96.1) γ
PD 22	3B	<i>Pseudomonas putida</i>	<i>Pseudomonas</i> sp.; JG35 + U1-AG16; AM071376 (98.1)	<i>Pseudomonas mandelii</i> AF058286 (96.3) γ
PD 26	2B	<i>Pseudomonas fluorescens</i>	<i>Pseudomonas</i> sp.; FTL216; AF529090 (97.9)	<i>Pseudomonas mandelii</i> AF058286 (94.6) γ
PD 30	ND	ND	<i>Pseudomonas</i> sp. BE3dii; AY263472 (98.5)	<i>Pseudomonas mandelii</i> AF058286 (98.2) γ
PD 1	2A	<i>Pseudomonas fluorescens</i>	<i>Pseudomonas</i> sp. HF3/S21027; AY747591 (99.6)	<i>Pseudomonas migulae</i> AF074383 (97.8) γ
PD 17	1B	<i>Pseudomonas fluorescens</i>	<i>Pseudomonas</i> sp. R1lenr; AY263479 (99.8)	<i>Pseudomonas migulae</i> AF074383 (97.1) γ
PD 12	3B	<i>Rhizobium radiobacter</i>	<i>Sinorhizobium</i> sp.; BK1; AJ012210 (100.0)	<i>Sinorhizobium fredii</i> X67231 (96.9) α

^aSource of soil–Soil identification Number (1, 2 or 3) followed by horizon of sample (A or B).^bND = not determined.

Table 2. Denitrification genes amplified from grouped soil isolates

Strain type	Nitrite reductase		Nitric oxide reductase		Nitrous oxide reductase <i>nosZ</i>
	<i>nirS</i>	<i>nirK</i>	<i>cnorB</i>	<i>qnorB</i>	
<i>Pseudomonas</i> spp. ^a (19) ^b	+ (13) ^b	–	+ (19)	–	+ (19)
<i>Pseudomonas chlororaphis</i> (2)	–	+ (2)	+ (2)	–	+ (2)
<i>Achromobacter</i> spp. (4)	–	+ (4)		+ (4)	+ (4)
<i>Bosea</i> spp. (4)	–	+ (4)	+ (4)	–	+ (4)
<i>Sinorhizobium/Ensifer</i> spp. (2)	–	+ (2)	+ (2)	–	+ (2)

^aAll *Pseudomonas* species from this study with the exception *Pseudomonas chlororaphis*.

^bNumber of isolates in each group and number of positive PCR results obtained.

90% of isolates amplified a ~450 bp region of the *cnorB* gene, which is found in the majority of known denitrifiers [6]. Only isolates identified as *Achromobacter* spp. amplified *qnorB* gene fragments (~640 bp). This is consistent with the information in [6], with *Alcaligenes* (the former strain designation of *Achromobacter*) strains represented amongst *qnorB* denitrifiers. Nitrite reductase gene fragments (*nirS* (890 bp) and *nirK* (~450 bp)) were amplified from the majority of strains isolated, although single bands of the appropriate size were not amplifiable from several strains (PD 2–5, 16, 30–31). PCR reactions were repeated and conducted at several annealing temperatures to amplify genes from these strains, but single bands of the correct size could not be obtained. It is probable that the strains have active nitrite reductase genes, given positive results obtained for denitrification, but it is unclear whether the PCR conditions used or greater gene polymorphism are responsible for the lack of amplification. Wang and Skipper [40] were also unsuccessful in amplifying *nirS* from a range of denitrifier isolates using the primers designed by Braker et al. [5].

nirS gene fragments were amplified from all *Pseudomonas* isolates except isolate PD 14, most closely related by 16S rDNA to *Pseudomonas chlororaphis*, from which *nirK* was amplified. Sequence analysis of *nirS* fragments from positive reactions showed high similarity to *Pseudomonas* sp. including *Pseudomonas fluorescens* and *Pseudomonas aeruginosa*, and many other uncultured clones (data not shown).

nirS and *nirK* genes have previously been shown to be present in closely related species of *Pseudomonas* [5]. Sequence analysis of the *nirK* gene from PD 14 indicated almost complete identity with *nirK* from *Achromobacter xylosoxidans* and *Alcaligenes* spp. Other *nirK* sequences analysed (data not shown) confirmed sequence similarity with known denitrifier strains.

Phylogenetic analyses

The neighbour-joining phylogenetic tree prepared using isolated *nosZ* gene fragments and database

sequences is presented (Fig. 1). The majority of *nosZ* sequences grouped with other *Pseudomonas* spp., as expected from the 16S rDNA similarities obtained (96.9–100%). Although there are a number of well-characterised denitrifiers within this group (i.e. *Pseudomonas stutzeri*, *Pseudomonas denitrificans*), the isolates obtained here were highly similar (82.4–96%) to the *nosZ* gene of only one well-characterised strain, *Pseudomonas fluorescens* C7R12 (AF197468). Within the *Pseudomonas* group, *nosZ* sequence similarities ranged from 81.6–100% for the region analysed. High similarities were obtained between *nosZ* genes from our isolates and isolates/clones from extreme environments (i.e. DQ179258, isolated from hydrocarbon-contaminated soil sampled in Antarctica). Other *Pseudomonas* isolates had high similarities to sequences obtained from agricultural soils (AY325645) and acid forest soils (AY912909).

Isolates PD 18, 19, 23 and 24, identified as *Bosea eneeae* by 16S rDNA sequence, were grouped separately from the *nosZ* gene of the closest characterised isolate, *Bradyrhizobium japonicum* USDA110. No *nosZ* clones or isolates in the database are identified as *Bosea* spp., but clones with high similarity have been isolated from a range of arable soils [15,27,34], indicating the ubiquity of this sequence/strain type in agricultural systems. *nosZ* clones with high similarity to *Bradyrhizobium japonicum* were also found in bacterial community profiles from a range of long-term fertiliser treatments including cattle manure, sewage sludge and Ca(NO₃)₂ [15].

Isolates PD 7, 20, 25 and 27 formed a separate, but closely related branch to *nosZ* of *Achromobacter xylosoxidans*. 16S rDNA sequence analysis also indicated high similarity of these strains to *Achromobacter* spp. The *nosZ* gene from strain PD 12 was closely related to the *nosZ* of '*Achromobacter cycloclastes*', but there is no coinciding 16S rDNA sequence associated with this strain name and it does not cluster with other valid *Achromobacter* spp. 16S rDNA sequence analysis of this strain PD 12 indicated similarity to *Sinorhizobium fredii*. PD 29, whilst closely related to PD 12 by 16S rDNA sequence analysis (99.1% similarity), clusters separately when *nosZ* sequences are compared.

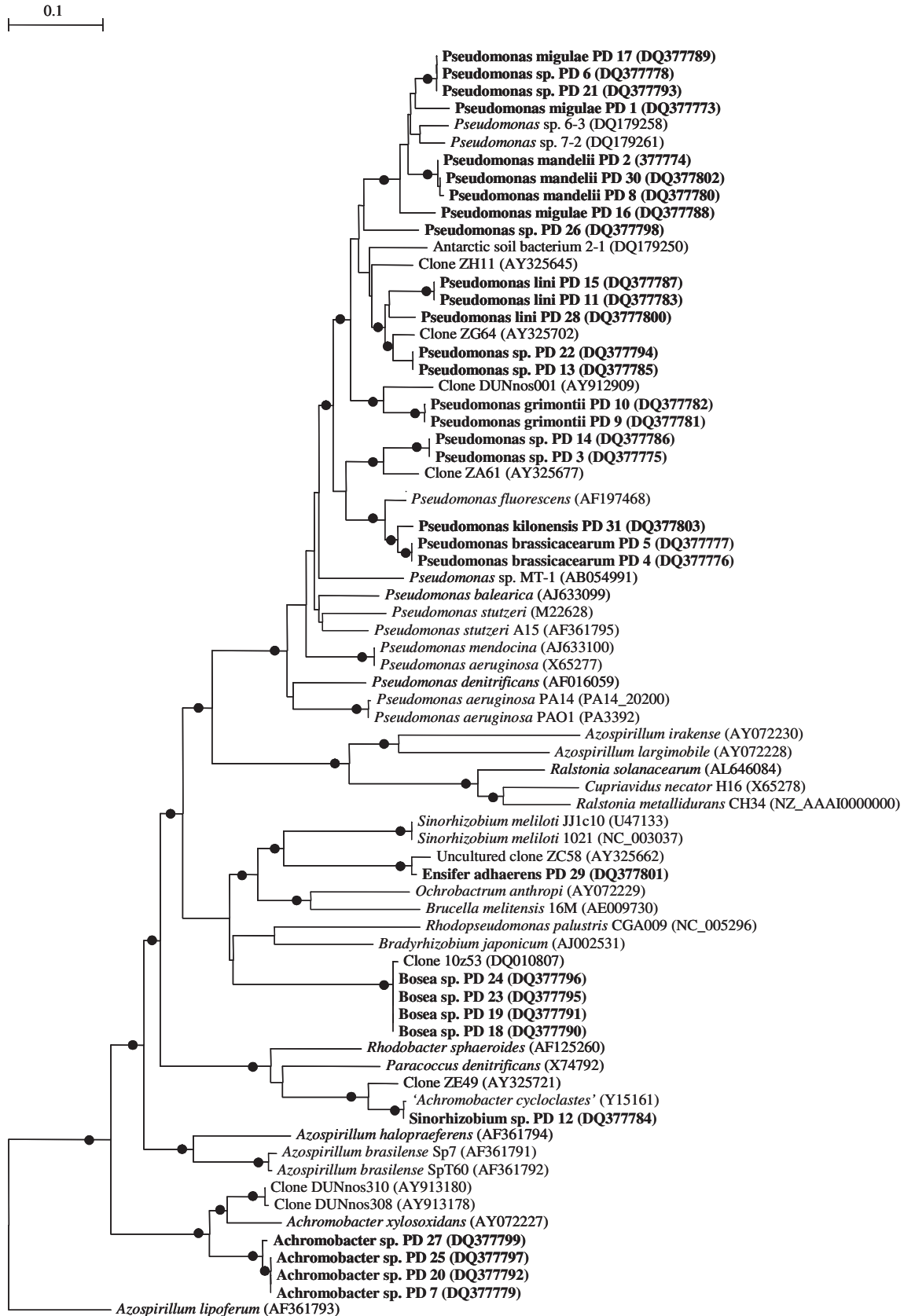


Fig. 1. Neighbour-joining phylogenetic tree of partial *nosZ* gene fragments. Genbank accession numbers are included for reference sequences used. The scale bar indicates the expected number of sequence changes per sequence position. Bootstrap values greater than 70% (500 replicates) are indicated by dots. The sequence of *Azospirillum lipoferum* (AF361793) was used as outgroup to root the phylogram.

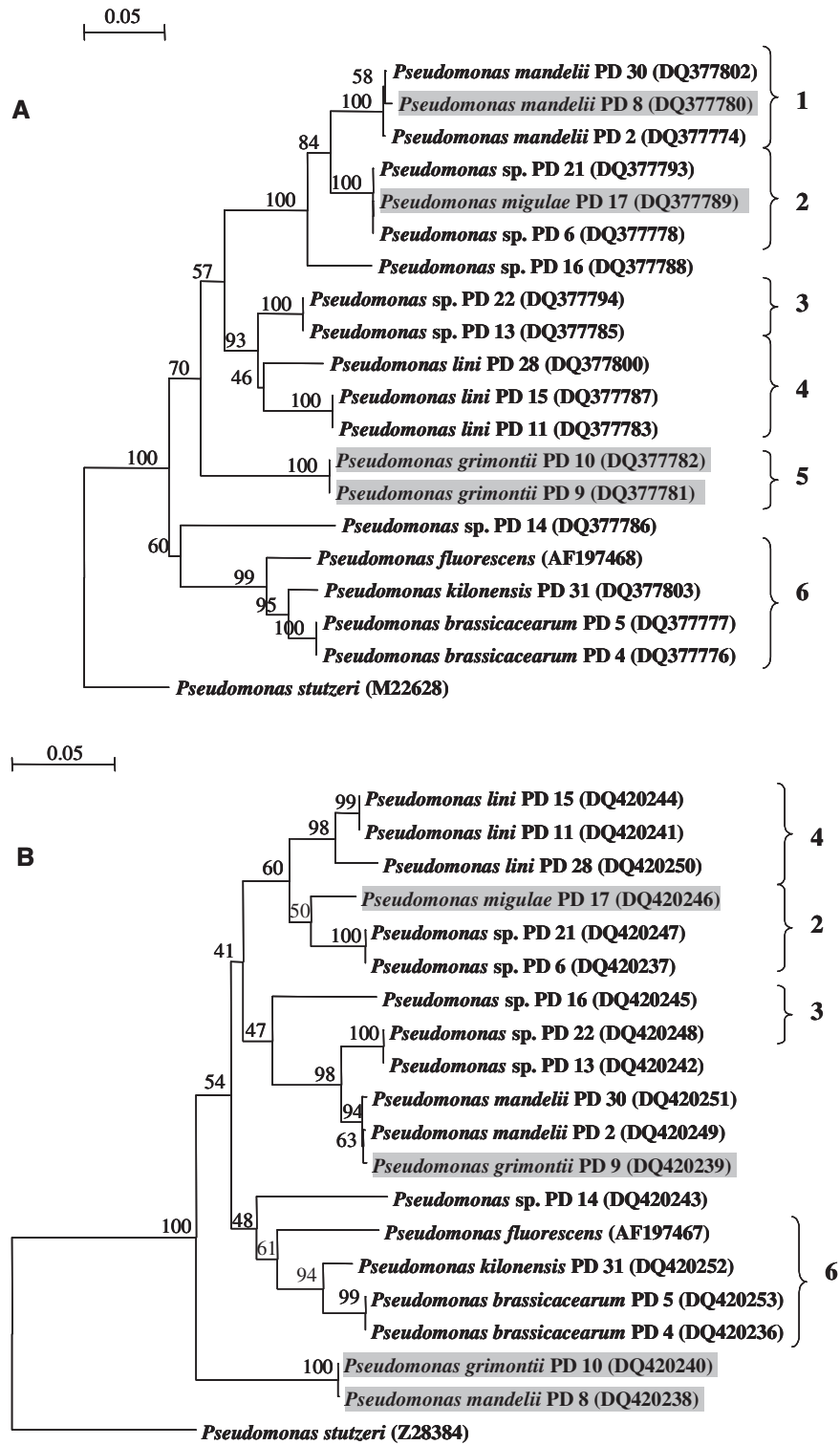


Fig. 2. Neighbour-joining phylogenetic trees of partial *nosZ* (A), *cnorB* (B) and 16S (C) genes from *Pseudomonas* sp. denitrifier isolates. *Pseudomonas stutzeri* ATCC 14450 genes (U26420: 16S rRNA, Z28384: *cnorB* and M22628: *nosZ*) were used as outgroups to root all three phylograms. *Pseudomonas fluorescens* C7R12 sequences (AM229082: 16S rRNA, AF197467: *cnorB* and AF197468: *nosZ*) were also included in phylograms as the closest cultured representative with all three genes sequenced. Bootstrap values are % of 500 replicates.

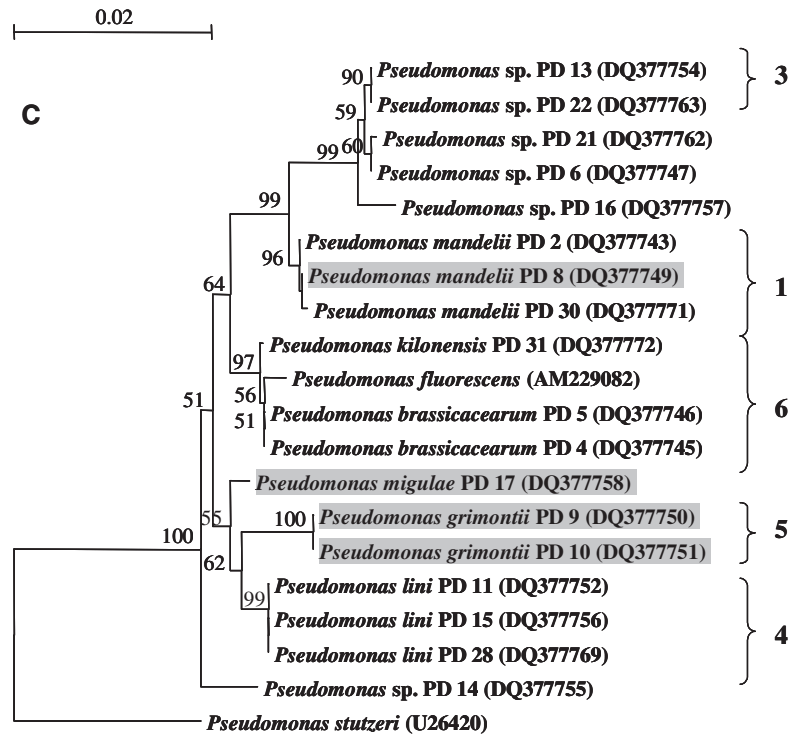


Fig. 2. (Continued)

Comparison of *nosZ*, *cnorB* and 16S rDNA phylogenies for *Pseudomonas* sp. isolates

A subset of *Pseudomonas* strains was compared based on *nosZ*, *cnorB* and 16S rDNA gene sequences (Fig. 2). Neighbour-joining trees were prepared for each gene and the distribution/clustering of strains based on this analysis was compared. Maximum-likelihood trees were also prepared for these sequences [16], for which the results obtained were similar to those presented here. The *nosZ* tree (Fig. 2A) shows strains clustering into 6 major groups. Analysis of *cnorB* (Fig. 2B) shows differences in tree topology, with PD 10 and 8 forming a separate new cluster and PD 9 joining the remainder of Group 1 sequences, PD 30 and 2. The *nosZ* groups are largely retained when 16S rDNA is analysed (Fig. 2C), except for *Pseudomonas migulae* PD 17 which shifts from Group 2 to Groups 4 and 5. Differences in topology of the phylogenetic trees here are potential evidence of horizontal gene transfer of denitrification genes to these isolates. Absolute topology of the trees is not clear from these analyses, but the minor groupings observed and changes amongst them are well supported.

With the large volumes of sequence fragments being generated from environmental cloning projects, there is still room for, and indeed need of, isolation and characterisation of novel denitrifying bacteria. The

isolates from this study have provided information regarding functional denitrifier gene sequences from a range of α -Proteobacteria. Further work in this area will involve use of these sequences to generate primers for quantification of denitrifier populations in situ. Isolated strains may [17] or may not [7] provide phylogenetic information regarding unknown groups identified in cloning experiments. In this study, although the denitrifiers isolated grouped with known denitrifying genera when analysed by 16S rDNA, more detailed species affiliation was obtained, particularly for the *Pseudomonas* spp. group. There were no major inconsistencies observed in phylogenetic affiliation of *nosZ* genes in these isolates, unlike the results obtained in [17] when comparing *nirS* and 16S rRNA phylogenetic trees. In that study, no clear correlation was obtained between the two gene phylogenies. Denitrification genes are not acquired or inherited in complete sets, and can be organised in varying ways on the chromosome or on megaplasmids [41]. Certain genes within the denitrification pathway may be more congruent with 16S rDNA phylogenies than others. Previous work [14] found no similarity between 16S rDNA and *nosZ* and *narG* (nitrate reductase) phylogenies in a group of fluorescent *Pseudomonas* spp. The isolates in that study belonged to a group of closely related fluorescent *Pseudomonas* spp., similar to the group of isolates identified in our work. When comparisons were made between all the major

groupings of strains in our study, denitrification genes and 16S rDNA showed similar phylogenies, but as noted above, topological incongruency was observed within the *Pseudomonas* group.

Acknowledgements

This project was funded by the GAPS programme of Agriculture and Agri-Food Canada.

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