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

C.E. Dandie[a], D.L. Burton[b], B.J. Zebarth[a], J.T. Trevors[c], C. Goyer[a,*]

[a]*Potato Research Centre, Agriculture and Agri-Food Canada, Fredericton, NB, Canada E3B 4Z7*
[b]*Department of Environmental Science, Nova Scotia Agricultural College, Truro, NS, Canada B2N 5E3*
[c]*Department of Environmental Biology, University of Guelph, Guelph, ON, Canada N1G 2W1*

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## 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.


***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–DQ158197 (*nirS*) and DQ518198–DQ518208 (*nirK*).

*Corresponding author. Tel.: +506 452 4851; fax: +506 452 3316.
*E-mail address:* goyercm@agr.gc.ca (C. Goyer).

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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 $N_2O$ 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 $KNO_3$ 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 $KNO_3$ 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