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Isolation, genetic and functional characterization of novel soil *nirK*-type denitrifiers

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ABSTRACT

Denitrification, the reduction of nitrogen oxides (NO_3^- and NO_2^-) to N_2 via the intermediates NO and N_2O, is crucial for nitrogen turnover in soils. Cultivation-independent approaches that applied nitrite reductase genes (*nirK*/*nirS*) as marker genes to detect denitrifiers showed a predominance of genes presumably derived from as yet uncultured organisms. However, the phylogenetic affiliation of these organisms remains unresolved since the ability to denitrify is widespread among phylogenetically unrelated organisms. In this study, denitrifiers were cultured using a strategy to generally enrich soil microorganisms. Of 490 colonies screened, eight *nirK*-containing isolates were phylogenetically identified (16S rRNA genes) as members of the *Rhizobiales*. A *nirK* gene related to a large cluster of sequences from uncultured bacteria mainly retrieved from soil was found in three isolates classified as *Bradyrhizobium* sp. Additional isolates were classified as *Bradyrhizobium japonicum* and *Bosea* sp. that contained *nirK* genes also closely related to the *nirK* from these strains. These isolates denitrified, albeit with different efficiencies. In *Devosia* sp., *nirK* was the only denitrification gene detected. Two *Mesorhizobium* sp. isolates contained a *nirK* gene also related to *nirK* from cultured *Mesorhizobia* and uncultured soil bacteria but no gene encoding nitric oxide or nitrous oxide reductase. These isolates accumulated NO under nitrate-reducing conditions without growth, presumably due to the lethal effects of NO. This showed the presence of a functional nitrite reductase but lack of a nitric oxide reductase. In summary, similar *nirK* genotypes recurrently detected mainly in soils likely originated from *Rhizobia*, and functional differences were presumably strain-dependent.

Introduction

Denitrification is the dissimilatory reduction of oxidized nitrogen compounds (NO_3^- and NO_2^-) to the gases nitric oxide (NO), nitrous oxide (N_2O), and dinitrogen (N_2) that are released concomitantly. NO and N_2O are important greenhouse gases, which support global warming and the destruction of the stratospheric ozone layer [20,24]. Denitrification processes in soils are a major source of atmospheric N_2O and soils contribute to approximately 57% of the global emissions [55]. The process is mainly driven by facultative anaerobic microorganisms, which use oxidized nitrogen compounds as alternative electron acceptors for energy production [86]. The reduction of NO_2^- to NO which is catalyzed by the enzyme nitrite reductase is the key step of denitrification. This step distinguishes denitrifiers sensu stricto from other nitrate respiring microorganisms, since the gases produced cannot be assimilated further by the organisms [86]. Two types of nitrite reductase exist, a copper- and a cytochrome cd_1-containing form which are encoded by the genes *nirK* and *nirS*, respectively. Both types occur as single copy genes in the same genus but not in the same microbial species. However, in one *Thauera* species two different functional copies of *nirS* were found [25] and duplicate copies of *nirS* were found in *Thiobacillus denitrificans* and *Dechloromonas aromaticum*, whereas *Magnetospirillum magneticum* had three *nirS* genes [42]. Nitrite reductase genes have been frequently used as marker genes to explore the functional group of denitrifiers in the environment because a 16S rRNA gene-based approach is not suitable due to the fact that the ability to denitrify is widespread among phylogenetically unrelated organisms [65]. Molecular studies of denitrifier communities in, for example, soil, ground water, activated sludge and marine habitats [14,26,31,33,37,38,72,81,82] have indicated highly diverse natural denitrifier communities. For soils, they have further shown a dominance of nitrite reductase genotypes that were derived from unknown, presumably as yet uncultured, organisms. For instance, phylogenetic analysis of cloned *nirK* gene fragments from soils of different locations world-wide and with distinct properties showed a preponderance of sequences that were consistently grouped within a single cluster. This cluster currently comprises more than 500 *nirK* sequences, mainly retrieved from soil [4,14,60,81,85]. However, the only sequence within this cluster

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originating from a cultured organism is the *nirK* gene from *Nitrosomonas* sp. TA-921i-NH4, which is a bacterial ammonia-oxidizer isolated from an estuary [15]. Other clusters within the *nirK* gene tree entirely lack sequences from cultured representatives but consist exclusively of environmental clone sequences. In contrast, sequences of a number of *nirK* clones from activated sludge were most closely related to *nirK* from cultured denitrifiers [32,59] suggesting that closely related functional genes might originate from organisms that are phylogenetically related. However, to infer a robust phylogenetic relationship of the organisms based only on their functional genes for denitrification is currently not possible, since horizontal gene transfer is likely to have occurred for denitrification genes [39,41]. Hence, cultivation studies are needed to unravel the phylogenetic affiliation of the large group of phylogenetically unrelated denitrifiers that are relevant in the environment and to study physiological properties of the organisms related to denitrification. A common strategy is to selectively enrich nitrate reducers, including denitrifiers from environmental samples, by using nutrient rich media and anaerobic conditions with nitrogen oxides as electron acceptors [73]. Although a variety of denitrifying bacteria were isolated successfully from soil [19,21,29] these cultivation attempts generally resulted in denitrifying bacteria with rather similar denitrification genotypes that showed little or no overlap with the *nirK* genotypes dominating in soils. Although results from molecular studies are also likely to be prone to bias, the fraction of the microbial community that can be taken in culture is known to be very small, thus leaving a large part of the community diversity undetected. Recently, however, with the development of advanced cultivation techniques, previously uncultured groups of bacteria that are widespread and dominant in soil were isolated successfully [23,41,43,61]. Hence, isolation approaches to overcome the 'great plate count anomaly' are promising for yielding isolates that are supposedly relevant for the functioning of natural communities.

In the present study, a molecular approach was first applied to assess the structure of the *nirK*-type denitrifier community of a typical temperate grassland soil. Then, cultivation of organisms showing the preponderant *nirK* genotypes within the overall NirK tree was attempted. Therefore, a strategy was used to generally enrich organisms that dominate the soil microbial community by mimicking the conditions prevalent in the environment (i.e. using aerobic conditions and low nutrients, as well as a comparably low isolation temperature). Resulting isolates were screened for the presence of a *nirK* gene coding for the key enzyme of denitrification and if they tested positive they were phylogenetically classified. The isolates were further characterized for the occurrence of additional genes coding for nitrogen oxide reductases and for their ability to denitrify.

Materials and methods

Cultivation and screening procedure

Grassland soil (alluvial soil) was sampled next to a river (Lahn) in Marburg, Germany (50°50′27, 37″N und 8°45′39, 81″E) in June 2006. A grab sample of the upper 10 cm of the soil was taken, transported to the laboratory and immediately homogenized and sieved at <2 mm. It had the following characteristics: pH ($CaCl_2$) 5.19, total nitrogen 0.31%, total carbon 3.19%, NO_3–$N_{(2\,M\,KCl)}$ 2.90 μg g^{-1} dry weight and NO_2–$N_{(2\,M\,KCl)}$ 3.43 μg g^{-1} dry weight. An aliquot (10 g) of the soil was suspended in 90 mL Ringer solution. In two parallel stepwise dilution series, aliquots (10 mL) of the soil suspensions were further tenfold diluted in 90 mL Ringer solution down to a dilution step of 10^{-10}. Aliquots (100 μL) of each of the dilutions 10^{-6} to 10^{-10} were spread plated on solid minimal medium, as described by Grosser et al. [30]. The medium was as follows: 2.5 mM NH_4NO_3; 1 mM $NaNO_3$; 0.75 mM Na_2SO_4; 4 mM $CaSO_4$; 0.25 mM K_2SO_4; 2 mM $MgCl_2$; 0.005 mM KH_2PO_4; 0.02 mM $FeSO_4$; 15 mM MOPS; nutrient broth (1:10,000 diluted [w/v]), gelrite 8 g L^{-1}, pH 7.1. Plates were incubated aerobically at 15 °C and checked weekly for growth of colonies. After a total incubation time of 4 weeks, colonies that had grown were screened for the presence of a *nirK* gene by PCR using gene specific primers and conditions described previously (Supplementary Table S1 [10,12]). Therefore, cells from single colonies were suspended in 50 μL of sterile double-distilled water and disrupted by freezing (−20 °C, 3 min) and thawing (100 °C, 3 min). Afterwards, cell debris was removed by centrifugation (16,000 × *g*, 4 °C, 10 min). The supernatant, containing the DNA, was then used as a template for the screening procedure (see below).

Colonies containing a *nirK* gene were re-streaked onto fresh plates and incubated again at 15 °C for 4 weeks. To purify the isolates, this procedure was repeated seven times. Purity of the isolates was proven by phase contrast microscopy and by sequence analysis of the 16S rRNA genes from several colonies of a given isolate (see below).

Since growth of the cultures in minimal medium was very slow, different media were tested to optimize growth conditions (nutrient broth [NB; 8 g L^{-1}], yeast extract mannitol medium [YEM; 10 g L^{-1} mannitol, 0.5 g L^{-1} K_2HPO_4, 0.2 g L^{-1} $MgSO_4$, 0.1 g L^{-1} NaCl, 0.4 g L^{-1} yeast extract, 2 mM KNO_3, pH 7.0] and R2A medium [0.5 g L^{-1} yeast extract; 0.5 g L^{-1} proteose–peptone; 0.5 g L^{-1} casamino acids; 0.5 g L^{-1} glucose; 0.5 g L^{-1} soluble starch; 0.3 g L^{-1} sodium pyruvate; 0.3 g L^{-1} K_2HPO_4; 0.05 g L^{-1} $MgSO_4 \cdot 7H_2O$; 0.5 g L^{-1} potassium nitrate; adjusted to pH 7.0]).

DNA extraction from soil and pure cultures

DNA extraction from 0.5 g field-fresh soil was carried out using the Fast®DNA SPIN Kit for soil (BIO101, La Jolla, CA), according to the manufacturer's instructions.

Cells from 2 mL of a pure culture suspension were collected by centrifugation at 16,000 × *g* at room temperature for 10 min. The pellet was resuspended in 500 μL sterile double-distilled water and the suspension was added to 0.5 g baked glass beads (diameter 0.17–0.18 mm, B. Braun Biotech International GmbH, Melsungen, Germany). Cell lysis was undertaken with a bead beater (FastPrep®-24, MP Biomedicals, Heidelberg, Germany) for 45 s with 6.5 m s^{-1}, and samples were centrifuged to remove cell debris (16,000 × *g*, 4 °C, 5 min). DNA in the supernatant was purified from proteins by phenol–chloroform extraction and subsequent ethanol precipitation [3].

The purity and quantity of the extracted DNA were determined by UV-spectrophotometry at 260 and 280 nm (NanoDrop® ND-1000 UV/vis-spectrophotometer, Peqlab Biotechnologie GmbH, Erlangen, Germany). The DNA was stored at −20 °C.

PCR amplification of 16S rRNA genes and functional genes

From the eight isolates that tested positive for *nirK*, 16S rRNA genes, as well as the functional genes *narG*, *napA*, *nirK*, *nirS*, *cnorB*, *qnorB* and *nosZ*, were PCR-amplified using gene specific primer pairs and conditions published previously (Supplementary Table S1). A typical amplification reaction mixture contained 25 pmol of each primer, 200 μM of each deoxynucleoside triphosphate (Roche Molecular Diagnostics GmbH, Mannheim, Germany), 400 ng μL^{-1} bovine serum albumin (Roche Molecular Diagnostics), and 1.25 U of REDAccuTaq LA DNA polymerase (Sigma–Aldrich Chemie GmbH, Steinheim, Germany) in 1× reaction buffer provided by the manufacturer. Template DNA (1 μL) and sterile water were added to a final volume of 25 μL reaction solution.