New molecular tools to detect *nosZ* and *nrfA* genes in soils: An expanded view of N_2O- and nitrite reduction among diverse bacterial populations

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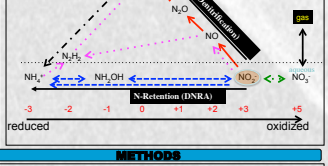
INTRODUCTION

Denitrification is thought to account for the main biological process mediating the reduction of nitrate to N_2O or N_2 in soil. Dissimilatory nitrate reduction to ammonia (DNRA) provides an alternate pathway and is catalyzed by a nitrite reductase encoded by *nrfA*. Testing the significance of DNRA was hampered previously by use of ineffective PCR primers that targeted only 77% of true *nrfA*. Conversely, genes facilitating denitrification are well-studied, particularly *nosZ*, the gene encoding nitrous oxide reductase. Recent whole-genome analysis of *nosZ*, however, revealed homologs distinct from those of known denitrifiers that are overlooked using existing molecular probes and include populations of non-denitrifiers. To improve our ability to detect a broader range of *nosZ* and address the significance of DNRA in soil, we designed PCR primers that effectively targeted new and diverse *nosZ* and *nrfA* genes. In two geomorphically distinct agricultural soils, we obtained clones that clustered into two main phyletic groups of *nosZ*: "typical" denitrifiers like *Pseudomonas stutzeri* and at least 27 novel "atypical" ones corresponding to diverse species like *Opitutus terrae* and the nitrite-ammonifier (ie. with DNRA) *Anaeromyxobacter dehalogenans*. Cloned *nrfA* sequences (n=95) amplified from both soils showed up to 30% divergence and were most similar to soil bacteria like *Anaeromyxobacter* spp. NO2- or NO3- fed enrichment cultures yielded evidence for atypical *nosZ* associated with *Dechlorimonas* sp. Sixteen soil isolates grown under nitrite selection included strains like *Bacillus* spp., *Ensifer adhaerens*, and *Pseudogulbenkiania subflava* with *nrfA* and/or atypical and typical *nosZ* detected. The results show a much higher diversity of *nosZ* and *nrfA* in soil than previously known. By adding new previously undescribed *nosZ* and *nrfA* sequences to existing databases and providing new strains that can serve as references, application of next generation sequencing technologies and metagenomics-based approaches can be expanded with improved functional gene annotations and identification of more diverse populations involved in N-cycling processes.



METHODS

Soil samples: Soils sampled across Illinois in 2006-2007 were used in initial PCR detection of the model population *Anaeromyxobacter dehalogenans* (harboring atypical *nosZ* & *nrfA*) and in 2010-2012 from Urbana (U) and Havana (H) for long term agroecosystem monitoring (see below for general description of field design and soil characteristics). All agricultural fields had long histories of commercial corn and soybean production.

DNA extraction and PCR analysis: DNA from all soil samples was extracted using a phenol-chloroform method (Tsai & Olson 1991 [ASM 57:1070]) with additional purification using Sephadex G-200 columns. The *nosZ* gene sequences were amplified from 25-50 ng soil DNA using specific primer pairs targeting conserved regions in the typical- and atypical *nosZ* genes. New atypical *nosZ* primers were designed to amplify ~1300 bp of the gene (NosZ661F Atyp1- 5'SGGCTATYGGCTWYGAYGA; NosZR Atyp1-5'RTGSARBGCSSWGCARAAG). Primers designed for targeting typical *nosZ* genes were NosZ661F Denit-5'GGSWWKACCAA; NosZR Denit2-5'DRTGCAKNGCRTGGCACGAA-3'. Primers targeting *nrfA* (nrfAF2aw/ nrfAR1, see section NrfA phylogeny and detection of *nrfA* in soils) amplified a ~250 bp product. Primers used to target *Anaeromyxobacter* 16S rRNA genes were 68-68f/447-465R (Petrie et al. 2003 AEM 69:7467).

Cloning and sequence analysis of *nosZ* and *nrfA* amplicons: The *nosZ* and *nrfA* gene PCR products amplified from Havana and Urbana soils were cloned and randomly selected for sequencing. Maximum likelihood phylogenetic analyses on the *nosZ* gene dataset was completed using the RAxML-VI-HPC BlueBlitz program v7.2.6 on the CIPRES computer cluster (http://www.phylo.org) and was based on a nucleotide alignment of 63 taxa and 1765 characters from each of the soil *nosZ* gene fragments analyzed and corresponding reference sequences from the database. The *nrfA* gene phylogeny included translated amino acids residues from the complete gene from 67 representative taxa from the FUNGENE database (http://fungene.cme.msu.edu) and was completed using MRBAYES. Soil clone sequences were assigned to specific clades based on sequence similarity to representative taxa. Sequences were assigned to specific clades on the basis of high PP and at least 30% mean, pairwise amino acid differences with a neighboring clade.

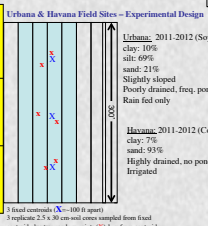
Community fingerprinting: Bacterial T-RFLP analysis was done using primers FAM-27 1492R to target 16S rRNA genes and FAM-labeled *nosZ* primers as described above, digested with HaeIII, HhaI, &BstUI, respectively. Fungal (F)-ARISA was analyzed using primers EF4f 5'GGAAGGGRTGTATTTATTAG and EF3r-FAM 5'TCCTCTAAATGACCAAGTTTG. Fragment data was analyzed using Genemapper v3.7 and statistical analysis was done using Primer v.6.

Isolation of nitrite-reducing bacteria: Serial dilations starting with 1g soil from Urbana or Havana were inoculated into mineral medium agar (pH 7) with or without 2mM ammonium, 1mM acetate or succinate, and 1mM nitrite. Colonies were selected and purified following growth at 25°C under an anaerobic N_2 atmosphere. Isolates were further tested on corresponding nitrite broth medium and 16S rRNA genes were sequenced for strain identities.

Parameter	Urbana (controls) N.M.S[a]	Havana (controls) N.M.S[a]
OM, TKN (%)	3.5, 0.15	0.5, 0.03
Moisture (%)	19.7	8.1
pH	5.8	7.7
N_2O Soil temp.	vary by depth	vary by depth
1- TRFLP		
2- ARISA		
atoxoZ-TFLP		
nosZ-TFLP		
OM, TKN (%)	3.5, 0.15	0.5, 0.03
Moisture (%)	19.6	8.7
pH	6.0	7.7
N_2O Soil temp.	vary by depth	vary by depth
1- TRFLP		
2- ARISA		
atoxoZ-TFLP		
nosZ-TRFLP		
OM, TKN (%)	3.5, 0.15	0.5, 0.03
Moisture (%)	19.8	7.4
pH	6.2	7.7
N_2O Soil temp.	vary by depth	vary by depth
1- TRFLP		
2- ARISA		
atoxoZ-TFLP		
nosZ-TRFLP		

*values are single measurements in Nov. 2011 shown for representativ comparisons between field sites

Urbana & Havana Field Sites – Experimental Design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