Denitrifying Bacteria Isolated from Terrestrial Subsurface Sediments Exposed to Mixed-Waste Contamination

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In terrestrial subsurface environments where nitrate is a critical groundwater contaminant, few cultivated representatives are available to verify the metabolism of organisms that catalyze denitrification. In this study, five species of denitrifying bacteria from three phyla were isolated from subsurface sediments exposed to metal radionuclide and nitrate contamination as part of the U.S. Department of Energy's Oak Ridge Integrated Field Research Challenge (OR-IFRC). Isolates belonged to the genera Afipia and Hyphomicrobium (Alphaproteobacteria), Rhodanobacter (Gammaproteobacteria), Intrasporangium (Actinobacteria), and Bacillus (Firmicutes). Isolates from the phylum Proteobacteria were complete denitrifiers, whereas the Gram-positive isolates reduced nitrate to nitrous oxide. rRNA gene analyses coupled with physiological and genomic analyses suggest that bacteria from the genus Rhodanobacter are a diverse population of denitrifiers that are circumneutral to moderately acidophilic, with a high relative abundance in areas of the acidic source zone at the OR-IFRC site. Based on genome analysis, Rhodanobacter species contain two nitrite reductase genes and have not been detected in functional-gene surveys of denitrifying bacteria at the OR-IFRC site. Nitrite and nitrous oxide reductase gene sequences were recovered from the isolates and from the terrestrial subsurface by designing primer sets mined from genomic and metagenomic data and from draft genomes of two of the isolates. We demonstrate that a combination of cultivation and genomic and metagenomic data is essential to the in situ characterization of denitrifiers and that current PCR-based approaches are not suitable for deep coverage of denitrifiers. Our results indicate that the diversity of denitrifiers is significantly underestimated in the terrestrial subsurface.

Nitrate is among the most prevalent of groundwater contaminants that threaten drinking water resources on a global scale (48). Nitrate and uranium are priority cocontaminants at U.S. Department of Energy (DOE)-managed nuclear legacy waste sites where nitric acid was used for the extraction and processing of radioactive metals (47). Current remediation practices favor reductive immobilization of U(VI) catalyzed by indigenous microbial communities, along with natural attenuation and monitoring, and previous studies have indicated that U(VI) reduction does not proceed until nitrate is depleted (1, 18, 29, 47, 63). Conversely, uranium can be remobilized into groundwater through oxidation of U(IV) precipitates by intermediate compounds produced during microbial denitrification (53), and some subsurface microorganisms can catalyze the oxidation of U(IV) coupled to nitrate reduction (5). Thus, when nitrate is present as a cocontaminant, the fate and transport of uranium in the contaminated terrestrial subsurface is intimately linked to the presence and metabolic activity of denitrifying bacteria.

Denitrifying microbial populations have been extensively profiled in soils, wastewater treatment systems, and marine environments (16, 26, 37, 46, 58). However, microorganisms that mediate denitrification and the mechanisms or most important controls of the process remain understudied in terrestrial aquifers (20, 48). Relatively few denitrifying bacteria from the terrestrial subsurface have been isolated and physiologically characterized in detail (17, 55). Furthermore, attempts to correlate microbial community structure and abundance, as assayed by molecular analyses of functional denitrification genes, to biogeochemical measurements in the subsurface have not been successful (44, 65).

Due to the paraphyletic nature and broad taxonomic distribution of denitrifying organisms, rRNA gene sequences are not suitable for demonstration of a denitrifying genotype (45). Instead, functional genes coding for proteins that catalyze denitrification are common targets for characterizing denitrifying populations by molecular analyses. Despite some successes (55), the application of functional-gene analyses for characterizing denitrifying communities has been limited by small genetic databases and the disproportionate representation of environmental sequences without reference sequences from isolated organisms. Likewise, the amplification of func-
tional genes from confirmed, isolated denitrifying bacteria with commonly used primer sets has met with mixed success (21, 24–26, 57). Thus, the development of more-general primer sets capable of amplifying such gene targets from a wider range of organisms mediating denitrification remains a challenge. As a result of the limits of both cultivation-based and molecular approaches, the diversity, abundance, and distribution of denitrifying bacteria in the environment are likely underestimated. This study investigated denitrifying bacteria and denitrification genes in contaminated, terrestrial subsurface sediments with the following objectives: (i) enrichment, cultivation, and purification of novel denitrifying bacteria from the Oak Ridge Integrated Field Research Challenge (OR-IFRC) subsurface; (ii) cultivation-directed amplification and phylogenetic analysis of gene targets for denitrifiers in subsurface sediments and groundwater; and (iii) development of improved primer sets for the detection of denitrifiers in environmental samples. We sought to improve access to the cultivable diversity of denitrifiers in the subsurface by using simple carbon substrates and a wide range of nitrate concentrations and by minimizing the salt and major nutrient concentrations in a minimal synthetic groundwater medium. In addition, we sought to expand the known phylogenetic target range of functional-gene primers through a combination of genomic- and metagenomic-sequence data mining coupled with analyses of these genes from the recovered isolates. We demonstrate that a polyphasic approach incorporating cultivation and genetic and metagenomic data is essential to the characterization of denitrifiers and that current broad-spectrum PCR-based approaches are not suitable for deep coverage of denitrifying microorganisms in the terrestrial subsurface.

MATERIALS AND METHODS

Sediment sampling. Sampling was conducted as part of the Department of Energy’s Oak Ridge Integrated Field Research Challenge (OR-IFRC) in Oak Ridge, TN, where the subsurface has been widely contaminated with a diverse array of mixed contaminants. The OR-IFRC site, managed by the Oak Ridge National Laboratory (ORNL), is located adjacent to the Y-12 industrial complex in Oak Ridge, TN. The contaminated area lies adjacent to a parking lot that caps three former waste ponds (S-3 ponds) which contained uranium and nitric acid wastes generated during nuclear weapon production (http://www.cvd.ornl.gov/orifice). Sediment cores were recovered from the saturated zone using a Geo-probe drill equipped with polyurethane sleeves lining the corer. A core from the most highly contaminated area (area 3, adjacent to the S-3 ponds) was recovered on 7 February 2008 (well FWB124; lat 35.97734171, long 84.27339281; 1.23 to 3.78 m below the ground surface), and a core from a less contaminated area was recovered on 12 September 2007 (borehole FB107; lat 35.97552296, long 84.2739979; 5.56 to 6.53 m below the ground surface). Cores were aseptically sectioned under strictly anoxic conditions in a Coy anaerobic chamber (Coy Laboratory Products, Grass Lake, MI) and stored anaerobically in gas-tight containers at 4°C prior to overnight shipment to Florida State University. Groundwater was sampled from well FWB124 (lat 35.97729757, long 84.27345838) within area 3 during a later part of a larger sampling operation on 13 May 2009. The subsurface of area 3 is generally acidic (i.e., with a pH below 4.0) and highly and variably contaminated with nitrate (may exceed 100 mM) and uranium (may exceed 200 μM), along with many other contaminants (for a list of identified contaminants, see reference 63). The area 2 experimental plot within the OR-IFRC site is located west and down-gradient of the S-3 pond site and has a circumneutral pH, though levels of nitrate (up to 26 mM) and uranium (0.5 to 4.5 μM) remain elevated relative to those in background areas (60). Methods and results from the geochronological characterization of the subsurface sediments used as inocula for our cultures are presented in the text and Table S1 in the supplemental material.

Enrichment and isolation of denitrifying bacteria. A minimal synthetic groundwater medium (SGWM) with trace element and vitamin mixes was prepared and dispensed according to Widdel and Bak (62), with the modifications of reducing the overall salt concentration (see below) and omitting resazurin, selenite, and tungstate. Unless otherwise indicated, this medium was used for all cultivation experiments. The medium contained the following components per liter: NaCl (100 mg), NH₄Cl (100 mg), KH₂PO₄ (50 mg), KCl (10 mg), MgCl₂·6H₂O (40 mg), CaCl₂ (40 mg), NaHCO₃ (2.5 g), trace element solution (TES; 1 ml), vitamin B₁₂ (1 ml), vitamin mix (1 ml), and thiamine (1.0 μl). The medium was cooled after being autoclaved under strictly anoxic conditions and was then saturated with N₂-CO₂ (80:20), resulting in a final pH of 6.9.

Four different concentrations of nitrate (0.5, 1.0, 10, and 25 mM) were used with four carbon sources (10 mM acetate; 10 mM lactate; a mixture of acetate, propionate, and butyrate at 10 mM each; and 20 mM ethanol), resulting in 16 enrichment conditions in SGWM. Each enrichment was performed in 60-ml capacity serum vials and contained 30 ml of completely anoxic SGWM, as described above. Each serum vial was inoculated with contaminated sediment to a final concentration of 10% (wt/vol) and incubated at 30°C in the dark. Growth was monitored by measuring the optical density of the medium at a wavelength of 600 nm (OD₆₀₀) using the NanoDrop ND-1000 spectrophotometer (Thermo Scientific, Wilmington, DE) and by colorimetric measurement of nitrate (10). Transfers to fresh medium were performed every 10 days using a 10% inoculum (vol/vol). After four successive transfers, isolation of distinct denitrifying bacteria was conducted by plating on agar medium composed of SGWM and 10 mM HEPES (Fisher Scientific, Fairlawn, NJ).

Growth rates and nitrogen gas production. Plates were screened to assess growth and nitrate utilization rates. Isolates were transferred to SGWM containing 1 mM nitrate and supplemented with the electron donor that produced the maximum growth. Lactate (10 mM) was used as the electron donor for Bacillus, Alphia, and Intrasporangium isolates, whereas acetate (10 mM) and ethanol (20 mM) were used for Rhodanobacter and Hyphomicrobium isolates, respectively. Nitrate was omitted from control cultures to test for fermentative growth. For gas measurement, mid-log-phase cultures were inoculated into SGWM at 1% (vol/vol) for Bacillus, Alphia, and Intrasporangium and at 5% (vol/vol) for Rhodanobacter and Intrasporangium, with nitrate (10 mM) and the appropriate electron donor. Cell densities and nitrate concentrations were measured as described above.

Concurrently, production of dinitrogen gas (N₂) and nitrous oxide (N₂O) was studied under denitrifying conditions (anoxic, nitrate amended) by growing representative isolates in SGWM amended with 15N-enriched NO₃⁻ (98 atom% 15N in a solution from Diversitron Isotope Laboratory, Andover, MA) and maintained at 3% H₂ in N₂ at room temperature. Pure cultures of the strains were acquired by repeatedly transferring morphologically distinct colonies to fresh plates. To test for aerobic growth, strains were inoculated in SGWM medium with the appropriate electron donor and incubated aerobically at 30°C. The purity of each culture was tested using multiple restreaks, as well as by colony PCR amplification with bacterial small-subunit (SSU) rRNA gene primers and subsequent direct sequencing without cloning. Isolates were subsequently preserved at –80°C in 20% anaerobic glycerol.

DNA extraction, PCR amplification, primer design, and gene sequencing. Genomic DNA (gDNA) was extracted from bacterial isolates, sediment, and groundwater samples using Mo Bio DNA isolation kits (Mo Bio Laboratories, Carlsbad, CA) according to the manufacturer’s instructions. Extracted genomic DNA (approximately 10 ng per reaction) was used as a template for small-subunit (SSU) rRNA gene amplification using the 27F and 1492R general bacterial primers (Table 1). PCRs were conducted using DreamTaq green DNA polymerase (Fermentas, Inc., Glen Burnie, MD), with a working concentration of 2.5 mM dNTPs, 1× reaction buffer, 100 nM each primer. Annealing temperatures and primer sequences for each primer set (functional-gene primer sets are numbered 1 to 7) are provided in Table 1. For rRNA gene amplification, 30 reaction cycles were employed. PCR yields were column purified using the UltraClean PCR clean-up kit (Mo Bio). For each represen-
sp. strain 2APBS1. Primers for nosZ subsequently confirmed with draft genome sequence data from water recovered in well FW106 in area 3 (22). These primer sequences were designed initially from metagenomic sequence data retrieved from ground-

(51). Primers for gene was generated using the online software Primer-BLAST at the NCBI

H. denitrificans

genetic contribution from the genus Hyphomicrobium denitrificans (45). Selected sequences were exported from ARB using a bacterial 50% consensus sequence alignment tool (15) and imported into the ARB software package

Representative nirK and nosZ genes from bacteria of the genus Rhodanobacter were designed from the draft genome of sp. strain A3151 (primer set 4 [64]). A primer set for this gene of Pseudomonas stutzeri (X56813).

c) According to the nirK gene of Pseudomonas stutzeri (X56813).

d) According to the nosZ gene of Pseudomonas fluorescens (AF197468).

tative isolate, multiple sequencing reactions were performed, the sequence data were aligned, and a composite sequence was determined using the software package Sequencer (Gene Codes, Ann Arbor, MI). The basic local alignment search tool (BLAST, 2) was used to identify closely related sequences and generate sequence similarity estimates. DNA from each of the isolates was subjected to PCR amplification (35 cycles) using primers targeting nitrite reductase (nirK and nirS) and nitrous oxide reductase (nosZ) genes. PCR amplification was conducted as described above, with various annealing temperatures (Table 1). PCR amplicons were cleaned as described above and sequenced directly. Primers specific to the nirK gene of Hyphomicrobium denitrificans were designed using a previously published sequence of H. denitrificans strain A3151 (primer set 4 [64]). A primer set for this gene was generated using the online software Primer-BLAST at the NCBI website (51). Primers for nirK genes from bacteria of the genus Rhodanobacter were designed initially from metagenomic sequence data retrieved from groundwater recovered in well FW106 in area 3 (22). These primer sequences were subsequently confirmed with draft genome sequence data from Rhodanobacter sp. strain 2APBS1. Primers for nosZ genes from bacteria of the genus Rhodo-

nirK5R GCC TCG ATC AGR TTR TGG 1040–1023

d) According to the nirK gene of Hyphomicrobium denitrificans (AB076600).

e) According to the nosZ gene of Escherichia coli (E05133).

TABLE 1. PCR primers used in this study

<table>
<thead>
<tr>
<th>Primer set</th>
<th>Individual primer</th>
<th>Gene</th>
<th>Sequence (5′→3′)</th>
<th>Primer position</th>
<th>Annealing temp (°C)</th>
<th>Intended target</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>27F 1492R</td>
<td>rs</td>
<td>AGA GTT TGA TCM TGG CTC AG</td>
<td>8–27°</td>
<td>55</td>
<td>Bacteria</td>
<td>Lane (34)</td>
<td></td>
</tr>
<tr>
<td>1 nirK1F nirK5R</td>
<td>nirK</td>
<td>GGM ATG GTK CCS TGG CA</td>
<td>526–542°</td>
<td>56</td>
<td>Universal</td>
<td>Braker et al. (7)</td>
<td></td>
</tr>
<tr>
<td>2 FtaCu R3Cu</td>
<td>nirK</td>
<td>ATC ATG CTC GTG CCG CG</td>
<td>568–584°</td>
<td>50</td>
<td>Universal</td>
<td>Hallin and Lindgren (21)</td>
<td></td>
</tr>
<tr>
<td>3 MG763-F1aCu MG763-R3Cu</td>
<td>nirK</td>
<td>ATC CTG TGC GAG CGG CC</td>
<td>568–584°</td>
<td>73</td>
<td>Rhodanobacter</td>
<td>This study</td>
<td></td>
</tr>
<tr>
<td>4 HD-nirK F1 HD-nirKR1</td>
<td>nirK</td>
<td>CCA GCT CAA CCT TCT CGT TC</td>
<td>439–458°</td>
<td>55</td>
<td>Hyphomicrobium denitrificans</td>
<td>This study</td>
<td></td>
</tr>
<tr>
<td>5 nirS1F nirS6R</td>
<td>nirS</td>
<td>CCT AYT GGC CGC CRC ART</td>
<td>763–780°</td>
<td>61</td>
<td>Universal</td>
<td>Braker et al. (7)</td>
<td></td>
</tr>
<tr>
<td>6 nosZ2F nosZ2R</td>
<td>nosZ</td>
<td>CGC RAC GGC AAS AAG GTS MSS GT</td>
<td>1603–1625°</td>
<td>65</td>
<td>Universal</td>
<td>Henry et al. (23)</td>
<td></td>
</tr>
<tr>
<td>7 Rh-nosZ-F Rh-nosZ-R</td>
<td>nosZ</td>
<td>CCG CTG GGG TGT CAC CAA</td>
<td>303–320°</td>
<td>63</td>
<td>Rhodanobacter</td>
<td>This study</td>
<td></td>
</tr>
</tbody>
</table>

RESULTS

Isolation of novel denitrifying bacteria. Denitrifying bacteria capable of reducing nitrate to nitrous oxide or nitrogen gas were isolated from sediment obtained from the OR-IFRC site using a systematic enrichment strategy (Table 2, Fig. 1). From each enrichment, more than 100 colonies were recovered, and based on colony morphology and growth pattern, 21 colonies were selected for further analyses. Analysis of bacterial SSU rRNA gene sequences revealed that the characterized isolates belong to five genera within three phyla. SSU rRNA gene sequences from all of the recovered isolates from area 3 samples were nearly identical and were most similar (>98.1%) to the SSU rRNA gene sequence of Bacillus bataviensis, a phenol-
Table 2. Phenotypic and genotypic characterization of denitrifying isolates

<table>
<thead>
<tr>
<th>Genus (class or phylum) and isolatea</th>
<th>Isolation conditions</th>
<th>Denitrification b</th>
<th>SSU rRNA c</th>
<th>Primer set (GenBank accession no.)</th>
<th>GenBank accession no. for draft genome sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Electron donorb</td>
<td>Nitrate concnc (mM)</td>
<td>N2O production</td>
<td>N2 production</td>
<td>Aerobic growthd</td>
</tr>
<tr>
<td>Afipia (Alphaproteobacteria)</td>
<td>1NEL2*</td>
<td>Lactate 0.5</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>2LS1</td>
<td>Lactate 1</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>3AS3*</td>
<td>Acetate 10</td>
<td>Neg</td>
<td>Pos</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>4LS1</td>
<td>Acetate 25</td>
<td>Neg</td>
<td>Pos</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>4LS4</td>
<td>Lactate 25</td>
<td>Neg</td>
<td>Pos</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>4LS5</td>
<td>Lactate 25</td>
<td>Neg</td>
<td>Pos</td>
<td>—</td>
</tr>
<tr>
<td>Hyphomicrobium (Alphaproteobacteria)</td>
<td>1NE51*</td>
<td>Ethanol 0.5</td>
<td>Neg</td>
<td>Pos</td>
<td>VW</td>
</tr>
<tr>
<td></td>
<td>2NE51*</td>
<td>Ethanol 1</td>
<td>Neg</td>
<td>Pos</td>
<td>—</td>
</tr>
<tr>
<td>Rhodanobacter (Gamma-proteobacteria)</td>
<td>2AS1*</td>
<td>Acetate 1</td>
<td>Pos</td>
<td>Pos</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>2APBS1*</td>
<td>APB 1</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>3LS1</td>
<td>Lactate 10</td>
<td>Pos</td>
<td>Pos</td>
<td>—</td>
</tr>
<tr>
<td>Intrasporangium (Actinobacteria)</td>
<td>3APBS1*</td>
<td>APB 10</td>
<td>Pos</td>
<td>Neg</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>3AS1</td>
<td>Acetate 10</td>
<td>Pos</td>
<td>Neg</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>4LS1*</td>
<td>Lactate 25</td>
<td>Neg</td>
<td>Neg</td>
<td>—</td>
</tr>
<tr>
<td>Bacillus (Firmicutes)</td>
<td>1NL3A3*</td>
<td>Lactate 0.5</td>
<td>Pos</td>
<td>Neg</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>2NL3A3*</td>
<td>Lactate 1</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>AS31</td>
<td>Lactate 0.5</td>
<td>—</td>
<td>—</td>
<td>—</td>
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<tr>
<td></td>
<td>AS32</td>
<td>Lactate 0.5</td>
<td>—</td>
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<tr>
<td></td>
<td>AS33</td>
<td>Lactate 0.5</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>AS34</td>
<td>Lactate 0.5</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>

a An asterisk indicates that the isolate is presented in a phylogenetic tree (Fig. 1).
b Electron donor concentrations: acetate, 10 mM; lactate, 10 mM; ethanol, 10 mM; acetate, propionate, butyrate (APB), 10 mM.
c Production of nitrous oxide or nitrogen gas was tested by gas chromatography, as described in Materials and Methods. Neg, negative; Pos, positive; —, not tested.
d Primer set number (Table 1) used for PCR amplification of the target gene. Amplification of 16S rRNA genes was performed using primers 27F and 1492R (Table 1). Neg, no amplification with any primer set for that functional gene.

degradation, aerobic, denitrifying firmicute (GenBank accession no. EU334358) (Fig. 1). The environmental sequence most similar to the SSU rRNA gene of Bacillus isolates was recovered from a uranium-contaminated waste pile (>99.2% similarity to GenBank accession no. AM292610) (Fig. 1).

The isolates recovered from area 2 subsurface sediment enrichments belonged to the genera Afipia, Hyphomicrobiurn, Rhodanobacter, and Intrasporangium. The Hyphomicrobiurn isolates showed high SSU rRNA gene sequence similarity (>99.3%) to a known denitrifier, H. denitrificans strain DSM1869 (GenBank accession no. Y14308), and to an isolate capable of degrading organophosphate pesticides (99.7% similar to Hyphomicrobiurn sp. strain LMAP-1, GenBank accession no. FJ549004). Sequences from Intrasporangium isolates were nearly identical (>99.4% similarity) to the SSU rRNA gene sequence of Intrasporangium calvum, an aerobic actinomycete that has not been well characterized (32, 40), and to an environmental sequence recovered from a clone library generated from OR-IFRC area 2 sediment (>99.4% similarity to GenBank accession no. DQ125903 [8]). The capacity for denitrification has not been shown previously in the genus Intrasporangium or the family Intrasporangiaceae (54). Some members of the family Intrasporangiaceae are capable of nitrate reduction to nitrite but not denitrification (31). An analysis of the draft genome of Intrasporangium sp. 4LS1 revealed the presence of a partial denitrification pathway including nitrate, nitrite, and nitric oxide reductase genes. No nitrous oxide reductase gene was found, consistent with the lack of N2 production by these isolates (Table 2), but a final determination awaits complete genome sequence data.

Isolates from the genus Afipia belong to the Bradyrhizobium, Agromonas, Nitrobacter, and Afipia (BANA) cluster of the class Alphaproteobacteria (52) and have SSU rRNA gene sequences that were nearly identical (>99.7% sequence similarity) to those of Afipia felis (GenBank accession no. AY513503), a cat pathogen (6), and to an uncultured rhizosphere organism (GenBank accession no. AJ863320). The BANA cluster includes methylo trophs (42) and organisms capable of heterotrophic nitrate assimilation (41). Some representatives of the genus Afipia are denitrifiers, including a soil isolate with 98% rRNA gene sequence similarity to the sequences of the isolates recovered in this study (GenBank accession no. AB162068).

Isolates from the genus Rhodanobacter were identical by SSU rRNA gene sequencing to a clone recovered from the area 2 subsurface at the OR-IFRC site (GenBank accession nos. GU233006, GU233007).
no. DQ125555 [8]) and highly similar (98.9%) to the SSU rRNA gene sequence of *Rhodanobacter thiooxydans*, an organism capable of thiosulfate oxidation and nitrate, but not nitrite, reduction (36). No validly described species of *Rhodanobacter* has been shown to denitrify (3, 14, 28, 36, 43, 61); however, Cheneby et al. (11) previously isolated several denitrifying bacteria from agricultural soil (GenBank accession nos. AY122296, AY122297, and AY122309), and based on their SSU rRNA gene sequences, these organisms belong within the genus *Rhodanobacter*. An analysis of the draft genome of *Rhodanobacter* sp. 2APBS1 revealed the presence of a complete denitrification pathway including nitrate, nitrite, nitric oxide, and nitrous oxide reductase genes.

**Growth rates and production of gaseous nitrogen by bacterial isolates.** Representatives of the five novel taxa were tested for nitrate consumption and growth under denitrifying (i.e., anoxic, nitrate-amended) conditions. Denitrification capability was demonstrated by measurement of nitrous oxide and nitrogen gas production. All isolates were capable of converting nitrate to gaseous nitrogen species and grew optimally when ethanol (*Hyphomicrobium*), lactate (*Afipia*, *Bacillus*, and *Intrasporangium*), or acetate (*Rhodanobacter*) was used as the sole carbon substrate and electron donor. Little or no growth was observed in the absence of nitrate. Growth rates of the isolates diverged significantly, and nitrate removal rates mirrored cell growth (Fig. 2).

**FIG. 1.** Bootstrapped (1,000 iterations) neighbor-joining tree of denitrifying isolate SSU rRNA gene sequences. Isolate sequences recovered in this study are in bold, with additional genetic and phenotypic properties provided in Table 2. Nodes supported by bootstrap values greater than 70% are indicated by numeric values, and nodes supported by Bayesian analysis, with posterior probability values greater than 95%, are indicated with gray circles. The scale bar represents 0.02 substitutions per nucleotide position.
Functional-gene analysis of isolates, environmental clones, and groundwater metagenomes. Functional genes involved in denitrification were PCR amplified from genomic DNA extracted from the isolates using primers targeting conserved regions of the nitrite reductase and nitrous oxide reductase genes. The results, reported as positive or negative for PCR amplification, are presented in Table 2. The isolates were tested initially for genes of two different forms of nitrite reductases (i.e., the cytochrome cd$_1$ gene, nirS, and the copper-containing nitrite reductase gene nirK) using several previously published primer sets (Table 1). Only genomic DNA from the *Afipia* isolates was amplified when either of the previously published nirK primer sets was employed (i.e., primer sets 1 and 2 in Table 2). Nitrite reductase gene amplification using published nirS primers was not successful for any of the isolates (primer set 5 in Table 2). Based on a known sequence of *nirK* in *H. denitrificans* (64), a primer set for this gene was designed and successfully used to amplify the *nirK* gene from genomic DNA extracted from the *Hyphomicrobium* isolates (primer set 4 in Table 2). The gene sequence from *Hyphomicrobium* sp. strain 1NES1 (GenBank accession no. GU814013) was 88% identical (with 95% positive matches) in its inferred amino acid sequence to the *nirK* product sequence of *H. denitrificans* (GenBank accession no. AB076606). Nitrous oxide reductase genes were also amplified from both alphaproteobacterial taxa, using previously published primers (primer set 6 in Table 2). However, nosZ gene amplifications using these primers were not successful for the Gram-positive *Rhodanobacter* isolates.

We were initially unable to amplify either nitrite or nitrous oxide reductase genes from the *Rhodanobacter* and Gram-positive isolates. Complete gene sequences, when present, were recovered from draft genome sequences of *Rhodanobacter* sp. 2APBS1 and *Intrasporangium* sp. 4LS1. We attempted to design primers specific to *Rhodanobacter* denitrification genes by examining sequence data recovered from the draft *Rhodanobacter* genome and from metagenome analyses of acidic and circumneutral groundwater recovered from OR-IFRC site wells (FW106 and FW301, respectively [22]). No nitrite reductase genes were detected in the larger background well metagenome (JGI taxon object identifier [ID] 2007427000; ~106 Mb), while two putative *nirK* genes and no *nirS* genes were identified in the acidic groundwater metagenome from well FW106 (JGI taxon object ID 2006543007; ~9.5 Mb). The *nirK* genes from the groundwater metagenome were found on a small contig with no other genes (contig 763; gene object ID 2007098279 [Fig. 3]) and on a large contig (~568,000 bp) containing a complete denitrification pathway (contig 1060; gene object ID 2007101994 [Fig. 3]). Phylogenetic analyses showed that the unaffiliated *nirK* gene found on the small contig was highly similar to one of the two *nirK* genes recovered from the draft genome of *Rhodanobacter* sp. 2APBS1 (GenBank accession no. GU233006 [Fig. 3, cluster B]). The metagenome *nirK* gene located on the large contig in close proximity to other genes in the denitrification pathway was phylogenetically distant from the small contig gene and was most similar to *nirK* genes from *Archaea* (Fig. 3). Within the *Rhodanobacter* sp. 2APBS1 draft genome, a second *nirK* gene was identified (GenBank accession no. GU233007), but phylogenetic analyses revealed that this gene was highly divergent from any other *nirK* gene associated with *Rhodanobacter* and was most similar to the *nirK* gene detected within the genome of a *Verrucomicrobia* isolate, *Opitutus terrae* PB90-1 (Fig. 3). Using primers targeting cluster B *Rhodanobacter* *nirK* genes (primer set 3 in Table 1), we were able to amplify *nirK* genes from genomic DNA extracted from groundwater recovered from well FW106 and from genomic DNA extracted from sediment recovered from an area 3 contaminated sediment (borehole FWB124; clones K7 and K12 [Fig. 3]).

The reason for the lack of *nirK* gene amplification from some isolates is clearly revealed in an examination of sequence mismatches between isolate gene sequences and representative primer sequences (primer R3Cu [Fig. 3] and primer F1aCu [see Table S2 in the supplemental material]). These and other published *nirK* primers target only class I *nirK* genes (Fig. 3, cluster A). Such primers will likely miss some of the class I genes as well due to the presence of synonymous mutations in the primer sites (e.g., *Shewanella denitrificans* with 8 combined nucleotide mismatches with primer set 2 but no amino acid mismatches [Fig. 3 and see Table S2 in the supplemental material]). In the primer R3Cu (Table 1), the only inferred conserved amino acid is the histidine residue which serves as a type II copper atom ligand (59). Within the class II *nirK* genes, a high level of sequence diversity is noted, and the class II *nirK* primers developed herein (primer set 3) appear to be highly specific to certain *nirK* genes from the genera *Rhodanobacter*, *Burkholderia*, and *Ralstonia* (Fig. 3, cluster B). The clustering of these genes is strongly supported by bootstrap and Bayesian analyses. No amplification of the *nirK* gene was achieved for the *Bacillus* isolates, and we note that no such genes for the genus are currently available. Nitrite reductase genes from the *Firmicutes* do not all cluster together; for example, the *nirK* genes from *Symbiobacterium thermophilum* and *Geobacillus*
spp. are highly divergent (Fig. 3). The nirK gene recovered from Intrasporangium sp. 4LS1 clustered with nirK genes from other Actinobacteria (Fig. 3). We generated a phylogenetic tree based on nosZ gene sequences recovered in this study and from complete or near-completely gene sequences recovered from the JGI and GenBank databases (Fig. 4). Nitrous oxide reductase genes were recovered from all proteobacterial isolates but neither of the Gram-positive isolates. Partial nosZ gene sequences were amplified and sequenced from two Rhodanobacter isolates, and sequences were identical to the full gene recovered from the draft genome of Rhodanobacter sp. 2APBS1. Phylogenetic analyses revealed that the nosZ gene sequence from Rhodanobacter sp. 2APBS1 was most similar to the nosZ sequence recovered from the OR-IFRC acidic-groundwater metagenome. Together, these sequences clustered with sequences of other representative members of the gammaproteobacteria (Fig. 4). The target range of a selected primer set (primer set 6) was examined by in silico comparison of DNA and inferred amino acid sequences from each organism to the primer sequences (Fig. 4; see also Table S3 in the supplemental material). The inferred target range of this primer and other commonly used nosZ gene primers are genes from alpha-, beta-, and gammaproteobacteria (Fig. 4, cluster A).
Not all nosZ genes from proteobacteria are amplified by this primer set, as demonstrated, for example, by the lack of amplification from the *Rhodanobacter* isolates. Comparison of the *Rhodanobacter* sp. 2APBS1 nosZ gene sequence to primer set 6 revealed five mismatches with the forward primer (see Table S3 in the supplemental material) and none with the reverse primer (Fig. 4). Genes from other taxa, including the *Archaea*, have four to eight mismatches with the highly degenerate primer nosZ2R (a combination of 32 variants), and similarly high sequence divergence is observed at other priming sites (Fig. 4; Table S3).

**DISCUSSION**

The scope of denitrifying bacterial diversity and activity remains uncertain, and terrestrial subsurface environments are understudied in comparison to other ecosystems, such as soils or wastewater treatment systems. An improved understanding of denitrifying microbial communities is required in order to predict and control denitrification mechanisms for the remediation of contaminated groundwaters. A multifaceted approach, in which cultivation-based and molecular analyses were combined with genomic and metagenomic data mining,
was taken to examine the native denitrifying community at the OR-IFRC site. A total of 21 bacterial isolates, representing five genera (Afipia, Bacillus, Hyphomicrobium, Intrasporangium, and Rhodanobacter) within three phyla, were isolated and subjected to genetic and phenotypic analyses. All of the isolates reduce nitrate to gaseous end products, either N₂ or N₂O. The isolates are all facultative anaerobes, consistent with the highly variable redox conditions in the subsurface at the OR-IFRC site. This is the first report demonstrating denitrification activity of bacteria from the genus Intrasporangium. Furthermore, our analyses of structural and functional-gene sequences have revealed that members of the genus Rhodanobacter comprise a diverse community of bacteria that are circumneutral to moderately acidophilic and can have a very high relative abundance in some areas of the acidic source zone at the OR-IFRC site (9, 22). Although a single study reported that some isolates within the genus Rhodanobacter are capable of denitrification (11), members of the genus have not been routinely associated with denitrification activity. The data presented in this study and metagenomic data from the OR-IFRC site further indicate the denitrification potential within the genus Rhodanobacter (22), though these organisms have not been previously identified in functional denitrification gene surveys of the OR-IFRC site (55, 65). Due to the large number of mismatches between established denitrification gene primers and the gene sequences from Rhodanobacter spp., we were unable to amplify nitrite and nitrous oxide reductase genes from the Rhodanobacter isolates using these commonly used primer sets.

The lack of amplification of functional denitrification genes from many denitrifying bacteria isolated from agricultural soils and wastewater treatment systems has been reported (21, 24, 26, 57). Our initial, unsuccessful attempts to amplify denitrification genes from the Rhodanobacter strains isolated in this study highlight the fact that key organisms with denitrifying capabilities are not targeted with commonly used primer sets. Primers developed from genomic and metagenomic data enabled us to amplify nirK genes from Rhodanobacter isolates and directly from environmental genomic DNA. Comparison of these gene sequences to those from full-genome studies revealed that the amplification difficulties arise because the commonly used nirK gene primers target only the class I form of copper nitrite reductases (CuNiR), and this form has not been found in bacteria from the genus Rhodanobacter. Two clusters of CuNiR were previously established by Philippot (45), including cluster or class I genes generally assumed to encode soluble periplasmic proteins and class or cluster II genes which encode an outer membrane-bound form in some organisms (e.g., Neisseria gonorrhoeae [27]). Additionally, Philippot (45) demonstrated the presence of more-divergent sequences from Gram-positive bacteria and from an ammonia-oxidizing organism (Nitrosomonas europaea). Commonly used nirK gene primers have been designed to target class I CuNiR genes, and the current data set of class I sequences is composed largely of alphaproteobacterial sequences (7, 30). Due to large sequence differences present even in conserved primer sites (often, but not always, located in copper-binding domains), it is not possible to amplify class II nirK genes with class I primer sets (Fig. 3) (30). The class I primer sets are also unsuitable for targeting nirK genes from a third cluster of nirK genes that are divergent from both class I and II genes (Fig. 3) (45). This cluster includes highly divergent genes from Actinobacteria, from the firmicute genus Geobacillus, from Verrucomicrobia and a member of the class Gemmatimonadetes, and from ammonia- or nitrite-oxidizing proteobacteria (Nitrosomonas and Nitrobacter). Curiously, one of the two putative nitrite reductase genes identified in the draft genome of Rhodanobacter sp. 2APBS1 belongs to this “third cluster” of nitrite reductase genes and is most similar to genes from Opitutus terrae PB90-1 (Verrucomicrobia) and to Gemmatimonas aurantiaca (Gemmatimonadetes). Neither the O. terrae nor the G. aurantiaca isolates have been shown to denitrify (12, 66), and this leaves uncertainty about the function of this putative nirK gene within Rhodanobacter sp. 2APBS1. We note that some third-cluster nitrite reductase genes are likely functional, as indicated by denitrification activity in the Intrasporangium spp. recovered in this study (Table 2; Fig. 2) and in other Actinobacteria (e.g., Jonesia denitrificans [49]).

For environmental systems such as the OR-IFRC site, where the predominant organisms may contain class II nirK genes, community composition analyses based on functional-gene primers can produce inaccurate quantitation and characterization of the subsurface denitrifying community. This problem is not unique to the contaminated subsurface. Of great concern is the DNA sequence divergence among the recovered nirK genes, which places considerable restraints on the design of suitable, broad-spectrum primers. Part of this sequence divergence is common to functional genes as a result of synonymous mutations. For nirK genes, copper-binding residues appear to hold the greatest hope for locating conserved sequences, and we identified a potential priming location where two copper-binding residues are adjacent (see Table S4 in the supplemental material). Although this region is much more highly conserved across a wide range of class I and class II nirK genes than among commonly used primers, we observed up to six mismatches with known nirK genes, such as those from Actinobacteria, including Intrasporangium sp. 4LS1, recovered in this study. As a result of the remarkable sequence divergence in nirK genes, we believe that direct PCR amplification or quantitative PCR amplification using currently employed primer sets cannot be reasonably employed to explore the true diversity and abundance of denitrifying bacteria in environmental systems.

For the contaminated subsurface, the limited metagenomic data from the site provide a PCR-independent indication that nirK genes are the dominant form in the highly contaminated source zone (two nirK and no nirS genes were detected in the acidic area 3 groundwater, and no nir genes were detected in a background well metagenome [22]). In addition, our results suggest that denitrifier abundance and diversity data based on nir gene amplification should be reevaluated. Likewise, the conventional wisdom that cytochrome cd₄ nitrite reductases (nirS) are environmentally more abundant than copper-containing reductases (13) should be reevaluated in the context of these findings. In parallel, we observed similar impediments to functional-gene detection with commonly employed nosZ gene primers, which exclusively target genes from alpha-, beta-, and gamma-proteobacteria, suggesting that previous denitrifier community studies using nosZ analyses most likely do not
eluicate the true diversity and abundance of denitrifying bacteria in the environment.

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