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# Denitrifying Bacteria from the Genus *Rhodanobacter* Dominate Bacterial Communities in the Highly Contaminated Subsurface of a Nuclear Legacy Waste Site

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**The effect of long-term mixed-waste contamination, particularly uranium and nitrate, on the microbial community in the terrestrial subsurface was investigated at the field scale at the Oak Ridge Integrated Field Research Challenge (ORIFRC) site in Oak Ridge, TN. The abundance, community composition, and distribution of groundwater microorganisms were examined across the site during two seasonal sampling events. At representative locations, subsurface sediment was also examined from two boreholes, one sampled from the most heavily contaminated area of the site and another from an area with low contamination. A suite of DNA- and RNA-based molecular tools were employed for community characterization, including quantitative PCR of rRNA and nitrite reductase genes, community composition fingerprinting analysis, and high-throughput pyrotag sequencing of rRNA genes. The results demonstrate that pH is a major driver of the subsurface microbial community structure and that denitrifying bacteria from the genus *Rhodanobacter* (class *Gammaproteobacteria*) dominate at low pH. The relative abundance of bacteria from this genus was positively correlated with lower-pH conditions, and these bacteria were abundant and active in the most highly contaminated areas. Other factors, such as the concentration of nitrogen species, oxygen level, and sampling season, did not appear to strongly influence the distribution of *Rhodanobacter* bacteria. The results indicate that these organisms are acid-tolerant denitrifiers, well suited to the acidic, nitrate-rich subsurface conditions, and pH is confirmed as a dominant driver of bacterial community structure in this contaminated subsurface environment.**

Terrestrial subsurface ecosystems comprise one of the largest habitats for microorganisms on Earth and represent a globally important resource of microbial diversity (26). Microorganisms in the terrestrial subsurface provide critical ecosystem services that include the mitigation of contaminants (33). Research on subsurface ecosystems has intensified over the past two decades, leading to important discoveries on the ecology, physiology, and phylogeny of subsurface microorganisms (14, 24). However, despite this excellent progress, the structure-function relationships of subsurface microorganisms remain largely uncharacterized. Attempts to correlate microbial abundance and community composition with critical physicochemical variables likely to control microbial metabolism have often been unsuccessful. New opportunities for understanding the response of microbial communities to environmental change are provided by a variety of robust molecular analyses, including deep sequencing. Few studies in contaminated subsurface environments have yet taken advantage of these new capabilities to address the spatial and temporal heterogeneity of microbial distribution in a systematic manner, particularly under *in situ* or natural attenuation conditions.

Our research has focused on subsurface ecosystems exposed to mixed waste contamination as a legacy of nuclear weapons production at sites managed by the U.S. Department of Energy (DOE) (44). At DOE sites, uranium and nitrate are often cocontaminants because of the use of nitric acid in the cleaning of equipment from uranium processing. For example, at the Oak Ridge Integrated Field Research Challenge (ORIFRC) site located in Oak Ridge, TN, decades of liquid waste disposal of nitric acid and uranium-bearing waste into unlined ponds (the former S-3 ponds) led to the creation of a subsurface contaminant source that persists even though the ponds have been drained and capped. Recent remediation research for such plumes has favored reductive immobilization of U(VI) catalyzed by microorganisms (5, 6, 30, 54). At the ORIFRC site, however, the high level of nitrate is a serious impediment to reductive immobilization, and in turn, has led to remediation strategies favoring monitored natural attenuation (31, 55). Despite intensive studies of the site, comprehensive site-wide microbial community analyses of native subsurface samples from the site are rare, and our understanding of such communities is often limited only to the control or pretreatment samples in bioremediation studies (2, 12).

In order to develop more effective remediation strategies for subsurface contamination and to determine the capacity of the native community to attenuate soluble contamination, the composition and metabolic potential of resident microbial communities needs to be better understood. The ORIFRC site also provides

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unusual environmental conditions that serve as a natural laboratory: namely, the site contains a gradient in contamination, including pH, nitrate, and uranium, from close to the source zone to down-gradient locations where subsurface conditions approach the background levels. Denitrification may play an important role in the subsurface ecology of the site due to the elevated levels of nitrate throughout the plume, which are often >10 mM in the near-source zone where the highest acidity is found. Furthermore, there is geochemical evidence for active denitrification at the site (Juske Horita, personal communication), and several groups have previously isolated denitrifying bacteria from the ORIFRC site subsurface (25, 47, 52).

The overall objective of this study was to systematically interrogate the response or adaptation of bacterial communities to the environmental contamination in shallow subsurface ecosystems across the plume gradient. Our hypothesis was that pH and nitrate would both exert strong selective pressure on the subsurface bacterial community across the contaminant plume.

## MATERIALS AND METHODS

**Groundwater sampling.** Sampling was conducted under the auspices of the U.S. Department of Energy's (DOE) Oak Ridge Integrated Field Research Challenge (ORIFRC) project at the Y-12 national security complex in Oak Ridge, TN, where the subsurface has been widely contaminated with a diverse array of mixed contaminants, including uranium and nitrate (http://www.esd.ornl.gov/orifrc/). Groundwater was collected in November 2008 and May 2009 from wells across the site, including near-source zone and down-gradient locations. Groundwater was also sampled from five wells near the contaminant source zone in October 2010. Water was collected separately for geochemical analysis (filtered), microbiological studies, including cultivation (unfiltered), and molecular analysis (collected on filters). When possible, two liters of water for each microbial sample were collected using a peristaltic pump to pass groundwater sequentially through two 142-mm Geotech filter holders (Geotech, Denver, CO) using a 3.0 $\mu$M Versapore prefilter and a 0.2-$\mu$M Supor sample filter (Pall, Port Washington, NY). Smaller quantities of water (0.5 to 2 liters) were filtered from some wells due to slow groundwater recharge. Groundwater was collected after purging the wells until pH, $E_h$, and temperature stabilized. Filters bearing biomass were stored frozen on dry ice in the field and then at −80°C until nucleic acid extraction.

Biogeochemical analyses were conducted on groundwater samples for pH, nitrate, nitrite, and ammonium. Nitrate plus nitrite ($NO_x$) and nitrite were reduced with vanadium (9) and iodide (22), respectively, and the resulting nitric oxide gas concentration was determined with a Thermo model 42i chemiluminescence analyzer (Thermo Fisher Scientific, WA). Nitrate was calculated as the difference between $NO_x$ and nitrite concentrations. Dissolved ammonium was determined colorimetrically (8).

**Sediment sampling.** Sediment cores were sampled from the saturated zone of the ORIFRC site using a Geoprobe equipped with polyurethane sleeves lining the corer. Cores were sampled from two boreholes from the most highly contaminated area (Area 3; adjacent to the former S-3 ponds) on 7 February 2008 (FWB124, 1.91 to 15.08 m below the surface), as well as a less contaminated area (Area 2; approximately 230 m southwest of the former S-3 ponds) on September 12, 2007 (FB107, 5.65 to 6.53 m below the surface). Sampling depths for Area 2 (A2) and Area 3 (A3) cores are shown in Table S2 in the supplemental material. Cores were aseptically subsectioned 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. Values for soluble and solid-phase geochemistry [i.e., pH, nitrate, sulfate, Fe(II), and Fe(III)] were determined as described previously (see supplemental material in reference 25). Uranium concentrations in sediment sections from FWB124 were measured using a Niton XLp 700 series environmental analyzer (Thermo Scientific).

**Extraction of nucleic acids from groundwater and sediment samples.** Genomic DNA was extracted from filtered groundwater samples using the Mo Bio PowerWater DNA isolation kit (Mo Bio Laboratories, Inc., Carlsbad, CA) according to the manufacturer's instructions. Genomic DNA was extracted from FB107 and FWB124 sediment sections using the Mo Bio PowerSoil DNA and UltraClean mega soil DNA isolation kits.

Total RNA was extracted from frozen filters shipped on dry ice from the ORIFRC site. Frozen filters were initially cut in half, and half was stored at −80°C. The remaining half of the filter was aseptically cut into small pieces which were distributed among six sterile, precooled 2-ml tubes and extracted using a protocol previously described by Chin et al. (15). Following RNA extraction, contaminating DNA was removed using a DNase I treatment (Ambion, San Francisco, CA). RNA extracts were verified to be free of contaminating genomic DNA (gDNA) by direct PCR amplification of the extracts. Reverse transcription reactions were performed using GoScript reverse transcriptase, following the manufacturer's protocol (Promega, Madison, WI), with the bacterial primer 907R (see Table S1 in the supplemental material).

**Quantitative PCR.** gDNA and cDNA from subsurface samples were used as templates for quantitative PCR (qPCR) amplification of bacterial small subunit (SSU) rRNA genes according to the protocol described by Nadkarni et al. (39) (see Table S1 in the supplemental material). A new primer set was developed to target SSU rRNA genes from bacteria of the genus *Rhodanobacter* (see Table S1 in the supplemental material), and these primers were used with the probe developed for domain-level bacterial qPCR analyses (39). The forward primer targets most known *Rhodanobacter* sequences and a broad but nonsystematic range of *Beta-* and *Gammaproteobacteria*. The reverse primer is highly specific to the family *Xanthomonadaceae* (21), and in combination, the two primers produce a primer set specific to the genera *Rhodanobacter*, *Dyella*, and *Luteibacter*. The *Rhodanobacter* primers were designed to have annealing properties similar to those of the general primers described by Nadkarni et al. (39), and the qPCR was performed as described therein, with the exception that the annealing temperature was raised to 63°C from 60°C. Standard curves were derived from PCR products generated by near-full gene amplification of SSU rRNA genes using the general bacterial primer set 27F/1492R (see Table S1 in the supplemental material).

In addition, a novel primer set targeting one of the genes encoding a copper-containing nitrite reductase (*nirK*) present in some members of the genus *Rhodanobacter* (25) was developed (see Table S1 in the supplemental material). The primer sites were chosen based on conserved regions of the *nirK* gene detected from the draft genome of *Rhodanobacter denitrificans* strain 2APBS1 (http://genome.jgi-psf.org/rho_2/rho_2.home.html) and a similar *nirK* gene detected in a metagenome generated from genomic DNA extracted from the ORIFRC site groundwater well FW106 (27). *In silico* analyses revealed that in combination, the two primers were exclusive to *R. denitrificans* strain 2APBS1 when compared to the GenBank nonredundant database. This primer set was used for qPCR using a SYBR green assay. Standard curves were derived from PCR products generated by partial gene amplification of the *nirK* gene from *R. denitrificans* strain 2APBS1 using the primer set RhNirK-B-14F and RhNirk-B-784R (see Table S1 in the supplemental material). All quantitative assays were performed using the ABI StepOne real-time PCR system with either SYBR green PCR master mix or TaqMan gene expression master mix (Applied Biosystems, Foster City, CA).

**TRFLP analysis.** Terminal restriction fragment length polymorphism (TRFLP) analyses were performed as previously described (1), with slight modifications. Bacterial SSU rRNA genes were amplified from DNA extracts with the primers 27F/1492, where the 27F primer was labeled at the 5′ end with 6-carboxyfluorescein (6-FAM; Applied Biosystems). PCR products were purified and quantified using a NanoDrop 1000 spectrophotometer (ThermoScientific), and 30 ng of PCR product from each reaction was digested with the restriction enzyme Bsh1236I (5′-CG/CG-3′) (New England BioLabs, Beverly, MA) for 3 h at 37°C. The digested

amplicons were recovered by ethanol precipitation, resuspended in formamide with a ROX-labeled size standard, run on an ABI 310 genetic analyzer (Applied Biosystems, Foster City, CA), and analyzed using Genescan software. For comparative analysis, all peaks were normalized to total peak area and small peaks representing <1% of the total area were excluded from further analysis. To analyze bacterial community structure, TRFLP profiles from each sample were transformed using $\log(x + 1)$ to reduce weighting biases of large peaks (43) and then analyzed using a Bray-Curtis similarity matrix, implemented within the software package Primer6 (16). The resulting resemblance matrix was analyzed and visualized using a multidimensional scaling (MDS) plot and a group average hierarchal cluster dendrogram. A one-way SIMPER test using Bray-Curtis similarity was used to determine which restriction fragments contributed to within-group similarities and between-group similarities.

To identify peaks in TRFLP profiles, the terminal fragment sizes of peaks were compared to a database of terminal fragment sizes generated *in silico* for the enzyme Bsh1236I. Predictions of TRF cut sites were generated using an algorithm developed within the software package Excel (Microsoft, Redmond, WA). A large data set of bacterial SSU rRNA gene sequences from the ORIFRC site were aligned using the Greengenes NAST aligner (20). The "Greengenes" alignments, fixed to an array of 7,682 positions, were used to identify the location of the forward primer (27F), and the Excel function "search" was used to identify the initial cut site for each enzyme within unaligned sequences.

**Pyrotag sequencing and phylogenetic analyses.** Genomic DNA extracted from 41 filtered groundwater samples was subjected to pyrotag sequencing analysis to examine the bacterial community composition and distribution across the watershed. These samples were sequenced by the Research and Testing Laboratories (Lubbock, TX), using primers targeting the V1 to V3 regions of the SSU rRNA gene (see Table S1 in the supplemental material). Sequence data were processed as described above, and at least 1,550 sequences from each groundwater sample were recovered and initially processed through the Ribosomal Database Project (RDP) Pyrosequencing pipeline (17). Sequences were clustered into operational taxonomic units (OTUs) using a 97% sequence similarity threshold using the complete-linkage clustering method. Subsequently, representatives of each OTU were classified using the RDP's classifier (51) and confirmed through subsequent BLAST analysis (3).

Genomic DNA extracted from sediment cores was also subject to pyrosequencing analysis using primers targeting the V4 region of the rRNA gene (see Table S1 in the supplemental material). The sequences from the sediment samples were pooled together and submitted simultaneously to the RDP Pyrosequencing aligner. The aligned sequences were then grouped as described above. The dereplication tool was used to identify representative sequences for each OTU, and the FASTA selection tool was used to retrieve these representative sequences from the total data set. Representative sequences from all OTUs (97%) identified were aligned using the Greengenes online software package (20). The returned alignments were imported into the software package ARB (37) containing a large database of near-full-length, aligned SSU rRNA gene sequences and an associated phylogenetic tree (19). The short sequences (approximately 250 bases) recovered from sediment samples were inserted into the full tree using the ARB parsimony option, implementing a bacterial 50% conservation filter (36). The phylogenetic tree was exported from ARB and analyzed using the online software package Unifrac (35). Principal coordinate analysis (PCoA) was performed as described previously (34), using weighted and normalized abundance data.

**Ordination of site geochemical and microbiological data.** Geochemical data (with the exception of pH) and qPCR data were transformed to meet assumptions of normality using the Ryan-Joiner normality test (46). For nitrate and oxygen, 4th-root transformations were used, while $\log_{10}$ transformations were used for uranium and all qPCR data. Transformed data were used to perform a principal component analysis (PCA), implemented within Primer6 and ultimately plotted in the software package Origin 8.5 (OriginLab Corporation, Northhampton, MA).

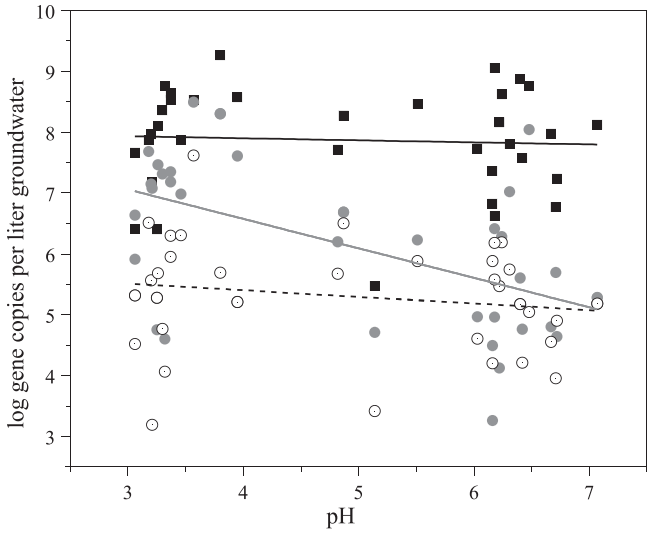


**FIG 1** Abundance of bacterial SSU rRNA and *nirK* genes in groundwater from across the contaminated watershed at the ORIFRC site. Bacterial SSU rRNA genes (black squares), *Rhodanobacter* SSU rRNA genes (gray circles), and *Rhodanobacter*-like *nirK* genes (open circles) were quantified in groundwater from samplings in November 2008 and May 2009 and plotted against groundwater pH. Linear regression lines for total bacterial SSU rRNA (solid black; slope −0.034, $R^2$ 0.003), *Rhodanobacter* SSU rRNA (gray; slope −0.484, $R^2$ 0.283), and *Rhodanobacter nirK* (black dashes; slope −0.109, $R^2$ 0.029) are indicated.

**Accession numbers.** The bacterial isolate *R. denitrificans* strain 2APBS1, isolated from Area 2 sediment of the ORIFRC site, has been accessioned at the German Collection of Microorganisms and Cell Cultures (DSMZ) under the accession number DSM 23569 and at the Japan Collection of Microorganisms under the accession number JCM 17641. The draft genome of the denitrifying *R. denitrificans* strain 2APBS1 can be found here: http://genome.jgi-psf.org/rho_2/rho_2.info.html. The denitrifying bacterial isolate *R. denitrificans* strain 116-2 (DSM 24678 and JCM 17642) was isolated from Area 3 groundwater of the ORIFRC site, and by DNA-DNA hybridization analysis (86 to 88% similarity) belongs to the same species as strain 2APBS1 (42). Phenotypic characteristics of both strains are described elsewhere (42). Sequence data were submitted to the European Nucleotide Archive (ENA) Sequence Read Archive (SRA) under the study accession number ERP001025.

## RESULTS

**Absolute and relative abundance of bacteria across the watershed.** The abundance of SSU rRNA genes from total bacteria and bacteria from the genus *Rhodanobacter* in ORIFRC site groundwater was assessed using quantitative PCR (qPCR) assays. Genomic DNAs recovered from filtered water samples from 21 wells during two sampling periods—November 2008 and May 2009—were assayed using qPCR for total bacteria and for bacteria from the genus *Rhodanobacter* (Fig. 1). In total, qPCR data from 35 sampling points were recovered, and these were characteristic of the broad range of subsurface geochemical conditions across the site, ranging from highly acidic and nitrate-rich close to the contaminant source zone (Area 3) to circumneutral with lower contaminant levels (e.g., lower nitrate) down gradient from the source zone (Area 2). Total bacterial rRNA gene abundance ranged from 2.98E+05 to 1.83E+09 copies per liter of groundwater, while *Rhodanobacter* rRNA gene levels ranged from 1.82E+03 to 3.13E+08 copies per liter of groundwater (Fig. 1). Although no

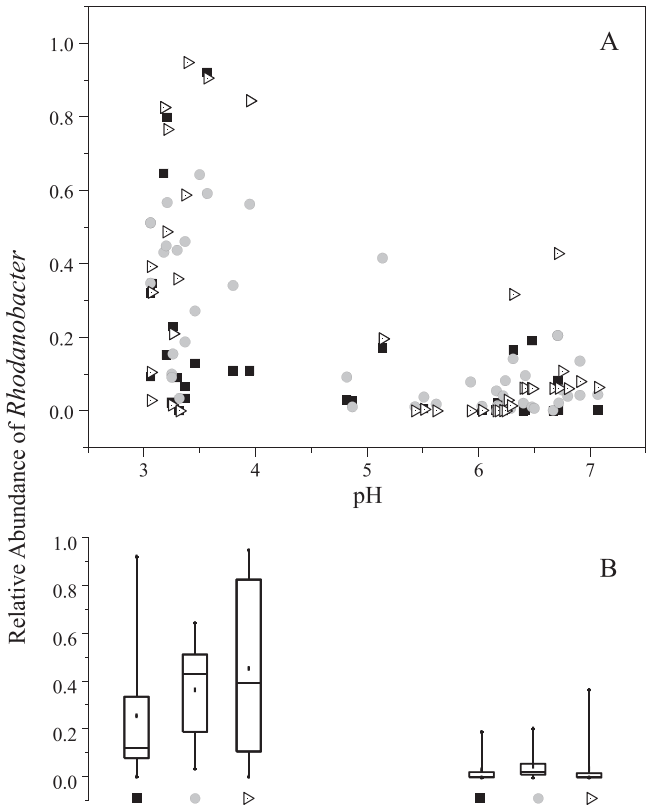


**FIG 2** Relative abundance of *Rhodanobacter* bacteria in groundwater across a pH gradient at the ORIFRC site. (A) The relative abundance of *Rhodanobacter* spp. as a proportion of the total bacterial community was estimated using quantitative PCR of total bacterial and *Rhodanobacter* SSU rRNA genes (black boxes), TRFLP analysis of bacterial SSU rRNA genes with diagnostic TRFs of *Rhodanobacter* (gray circles), and RDP classification of recovered bacterial SSU rRNA gene pyrosequences (open triangles). (B) Box plots of the relative abundance of *Rhodanobacter* SSU rRNA genes by pH as estimated by the three methods listed above. For each method, two plots were generated, one for groundwater samples with pH values below 4 and one for samples with pH values above 6. The box plot whiskers represent the minimum and maximum values. For each set of values, the horizontal line represents the median and the internal black dot represents the mean.

trend was observed between bacterial rRNA gene abundance and pH, *Rhodanobacter* rRNA gene abundance was inversely correlated with pH (Fig. 1). In the highly acidic near-source-zone samples, rRNA genes from *Rhodanobacter* species accounted for up to 92% of all detected rRNA genes as assessed by qPCR (Fig. 2A). Although *Rhodanobacter* rRNA gene abundance was highly variable, ranging over 5 orders of magnitude, the relative abundance of *Rhodanobacter* rRNA genes was significantly greater ($P < 0.004$ by independent *t* test) in groundwater samples with pH values below 4 than in samples with pH values above 6. In groundwater samples with pH values below 4, SSU rRNA genes from bacteria of the genus *Rhodanobacter* were on average 25.4% of total bacterial rRNA genes ($n = 16$), and they were 3.2% in groundwater samples with a pH above 6 ($n = 15$) (Fig. 2B).

We previously demonstrated that *Rhodanobacter* spp. have highly divergent gene sequences encoding copper-containing nitrite reductases (*nirK*), and we developed novel primer sets to amplify *nirK* genes from *R. denitrificans* strain 2APBS1 (25). Here, an additional primer set for quantitative analysis of this gene was developed (see Table S1 in the supplemental material). Using this novel primer set, *Rhodanobacter*-like *nirK* genes were detected in the ORIFRC site groundwater, with a range of 1.56E+03 to 4.14E+07 copies per liter of groundwater (Fig. 1). A slight but not significant decrease in measured *Rhodanobacter*-like *nirK* abundance was observed with increasing pH.

To investigate bacterial community composition and the distribution of major bacterial groups *in situ*, additional analyses were performed: terminal restriction fragment length polymorphism (TRFLP) analysis of bacterial SSU rRNA genes and pyrotag sequencing of SSU rRNA genes from multiple wells across the watershed (Fig. 2). Restriction digests using the enzyme Bsh1236I produced diagnostic peaks from *Rhodanobacter* SSU rRNA gene sequences in the size range of 205 to 215 bp. Some of this size variability is due to true size differences in the rRNA gene between different sequences of *Rhodanobacter*, while size estimation variability could also contribute to this range of fragment sizes. Although the SSU rRNA genes of other organisms can yield TRFs of 205 to 215 bases when restricted by Bsh1236I, TRFs in this range are highly diagnostic for *Rhodanobacter* at the ORIFRC site (data not shown). Each method (qPCR, TRFLP, and pyrosequencing) demonstrated the same general trend: the abundance of bacteria from the genus *Rhodanobacter* varied but was highest in the acidic groundwater closest to the source zone and much lower (both absolute and relative abundance) in circumneutral groundwater down gradient from the source zone (Fig. 2).

Additionally, a strong correlation between the relative abundance of *Rhodanobacter* SSU rRNA genes detected using qPCR and pyrosequencing was observed, with both methods producing nearly identical results for many samples (see Fig. S1 in the supplemental material). When comparing the percent abundance of *Rhodanobacter* rRNA genes to the relative abundance determined by TRFLP fingerprinting (the percentage of peak area accounted for by peaks from 205 to 215 bp), there was a strong correlation, though with high variability (slope 0.67; $R^2 = 0.43$), with the percent TRFLP area generally exceeding that of the qPCR relative abundance (data not shown). Ordination plots of the qPCR and TRFLP data demonstrated a strong clustering of bacterial communities from acidic samples, driven in large part by the high relative abundance of *Rhodanobacter* sequences in each of these samples (Fig. 3A and B).

Principal component analysis (PCA) was performed to investigate the effects of measured geochemical variables on gene abundances for groundwater samples (Fig. 3A). The percent abundance of *Rhodanobacter* bacteria was strongly correlated with uranium concentration and pH (Fig. 3A; also see Fig. S2A in the supplemental material). At the ORIFRC site, the groundwater uranium concentration is strongly, though not exclusively, dependent upon pH. For example, a linear regression of log-transformed uranium concentrations (parts per million [ppm]) plotted against pH gave a slope of −0.839 with an $R^2$ of 0.685 (see Fig. S2B in the supplemental material). Unlike the abundance and relative abundance of *Rhodanobacter* SSU rRNA genes, the absolute abundance of total bacterial SSU rRNA genes and *Rhodanobacter*-like *nirK* genes did not correlate strongly with any geochemical variables (Fig. 3A). Low-pH groundwater samples clustered together both in a PCA based on groundwater geochemistry and quantitative analysis of microbial gene abundances and in a nonmetric multidimensional scaling (NMDS) generated from bacterial TRFLP analyses (Fig. 3A and B). Although nitrate was

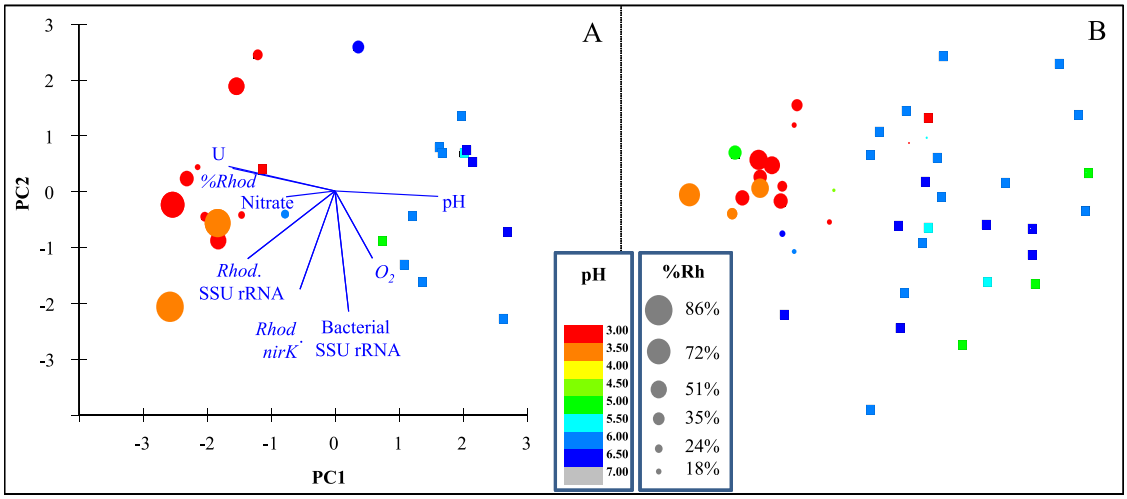


**FIG 3** Ordination of groundwater samples. (A) Principal component analysis of SSU rRNA and *nirK* genes and select groundwater geochemical variables. Geochemical and quantitative PCR data were transformed to meet assumptions of normality as described in the text. Vectors represent variables used to generate Euclidean distance. PC1, principal component 1 (*x* axis), explains 43.8% of the variation; PC2, principal component 2 (*y* axis), explains 19.6% of the variation. *Rhod*, *Rhodanobacter*. (B) Nonmetric multidimensional scaling (NMDS) plot of TRFLP-based groundwater bacterial community composition across the ORIFRC site. The TRF data from each sample were analyzed as described in the text, and the matrix was then displayed using NMDS in a 2-dimensional configuration (stress = 0.23). The relative abundance of SSU rRNA genes attributed to bacteria from the genus *Rhodanobacter* was estimated by fluorescence area derived from diagnostic TRFs, as described in the text. Both the PCA and NMDS bubble plots are color mapped by groundwater pH, and the relative abundance of *Rhodanobacter* SSU rRNA genes is indicated by the bubble size. Squares indicate data points where the abundance of *Rhodanobacter* bacteria is too low to be visualized.

weakly negatively correlated with pH at the site (see Fig. S2C in the supplemental material), nitrate concentration was not correlated with *Rhodanobacter* abundance at the site (see Fig. S2D in the supplemental material).

**Activity of *Rhodanobacter* bacteria in acidic groundwater.** As a proxy for the metabolically active bacteria, bacterial SSU rRNAs were quantified by qPCR of cDNA generated by reverse transcription of groundwater filter RNA extracts. General bacterial and *Rhodanobacter*-specific SSU rRNA assays were performed as described above. In all five acidic wells sampled in October 2010, *Rhodanobacter* rRNAs accounted for more than 47% of total bacterial rRNAs (Table 1). In two of the wells, *Rhodanobacter* bacteria were essentially the only active organisms detected, with more than 97% of total rRNAs attributable to members of *Rhodanobacter*. These findings were verified by direct sequencing of the qPCR products from these reactions (data not shown). Total bacterial rRNA levels ranged from 7.92E+06 to 2.38E+08 copies per liter of groundwater.

**Geochemical characterization of sediment samples.** Sediment cores from two boreholes were recovered from the site in September 2007 and February 2008. Sediment from borehole FWB124 (Area 3) was recovered from an area of the ORIFRC site subsurface that is heavily impacted by the contaminant plume from the former S-3 ponds. Cores were sectioned from depths ranging from 1.23 to 15.08 m below surface (mbs), and geochemical and microbiological data from each sampling depth are presented in Table S2 in the supplemental material. Sediment pH was circumneutral near the surface and decreased with depth. pH values over the interval from 5.77 to 13.51 mbs were from 3.2 to 4.0, with groundwater nitrate values ranging from 0.14 mM closer to the surface to 7.77 mM at depth. The deepest interval, 14.95 to 15.08 mbs, was highly contaminated with uranium (172 mg/kg) and nitrate (32.04 mM), but the pH was elevated (6.4) due to the presence of carbonate-bearing minerals at this depth (52). Uranium concentrations generally increased with depth and ranged from 4.8 to 196.7 ppm throughout the core. Levels of Fe(II) were low relative to those of total Fe (5% or below). Sediment from borehole FB107 (Area 2) was recovered from an area of the ORIFRC site subsurface that is less contaminated. Six core sections within the borehole were analyzed, from a depth of 5.56 to 6.53 mbs. Geochemical and microbiological data from each sampling depth are presented in

**TABLE 1** Quantification of bacterial and *Rhodanobacter*-specific rRNAs in acidic groundwater from Area 3

| Well | pH | Total *Bacteria* | | | *Rhodanobacter* | | | Relative abundance of *Rhodanobacter* (%) |
|---|---|---|---|---|---|---|---|---|
| | | Mean quantity (copies/liter) | SD | % SD | Mean quantity (copies/liter) | SD | % SD | |
| FW106 | 3.76 | 2.38E+08 | 1.86E+07 | 7.82 | 1.48E+08 | 8.35E+06 | 5.65 | 62.20 |
| FW128 | 3.60 | 7.92E+06 | 2.62E+05 | 3.30 | 3.74E+06 | 4.47E+05 | 11.93 | 47.28 |
| FW130 | 3.54 | 1.65E+07 | 2.80E+06 | 17.03 | 1.60E+07 | 2.77E+06 | 17.31 | 97.24 |
| FW115 | 3.43 | 5.06E+07 | 5.36E+06 | 10.59 | 5.01E+07 | 1.34E+06 | 2.68 | 98.97 |
| FW129 | 4.15 | 1.60E+08 | 3.79E+06 | 2.37 | 1.42E+08 | 3.46E+07 | 24.38 | 88.85 |

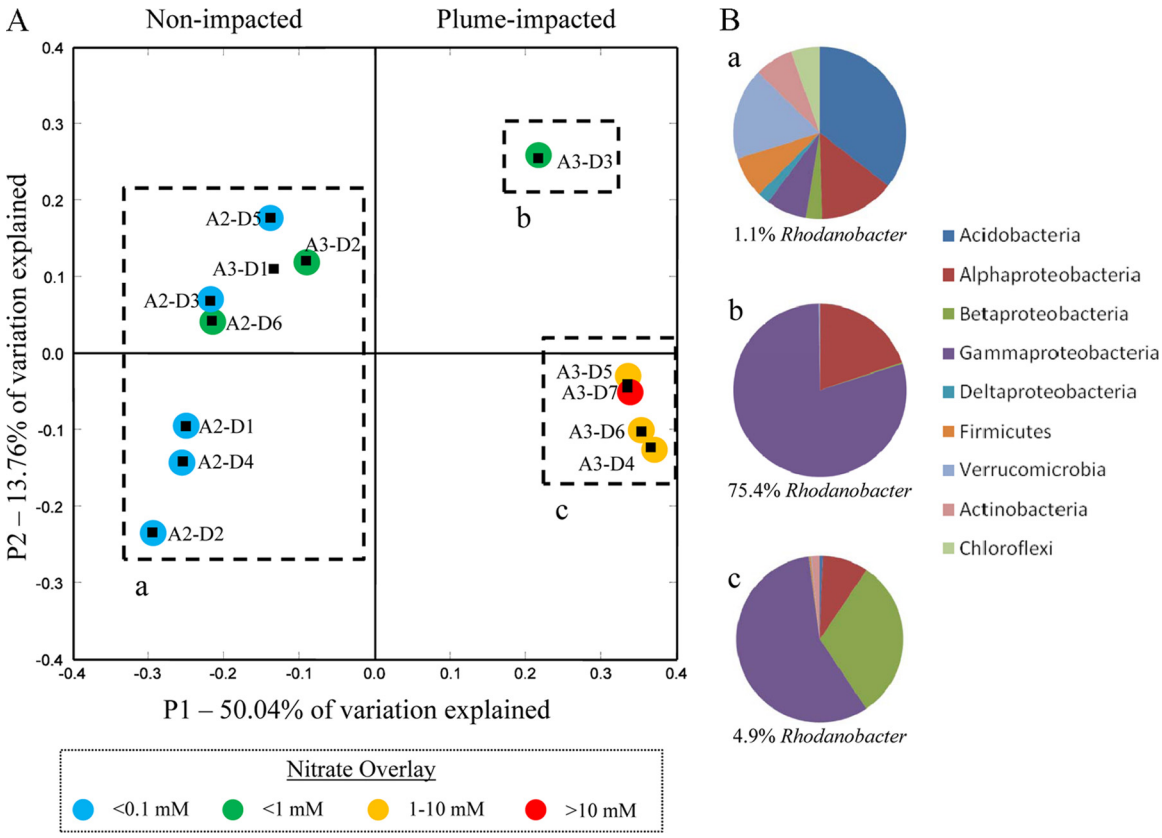


**FIG 4** Ordination of subsurface sediment samples with UniFrac. (A) Principal coordinate analysis (PCoA) of weighted UniFrac values calculated from an ARB parsimony insertion tree made with ~200 base sequence reads in the V4 region of the bacterial SSU rRNA gene. Sequences were clustered into OTUs with 97% similarity thresholds (4,898 defined OTUs), and the relative abundance of each OTU for each sample and depth was calculated. The figure shows a plot of the 1st (*x* axis) and 2nd (*y* axis) principal coordinate axes, explaining 50.04 and 13.76% of the variation, respectively. Each solid-square symbol represents a single sediment sample (see Table S2 in the supplemental material), and the concentration of nitrate in these samples is indicated by a color-coded circle. (B) Taxonomic affiliation at the phylum and class level of sequences from each defined cluster of samples (dashed boxes a, b, and c) in panel A. Sequences were classified using the Ribosomal Database Project (RDP) classifier, as described in the text.

Table S2 in the supplemental material. Pore water nitrate was below detection at all depths except the bottommost.

**Absolute and relative abundance of bacteria in subsurface sediments (FWB124).** The subsurface sediment profile across a gradient in contaminant chemistry was significantly different than that observed across the comparable gradient of contamination in groundwaters. Quantitative PCR analysis of bacterial SSU rRNA genes revealed a decrease of nearly 4 orders of magnitude in gene abundance per gram of soil across the contaminant gradient. In the circumneutral depths near the top of the FWB124 borehole (A3-D1 and -D2), bacterial SSU rRNA gene abundance ranged from 5.92E+07 to 1.93E+08 copies per gram of sediment, while at all contaminant-impacted depths below, gene abundance ranged from 2.85E+04 to 5.17E+04 per gram of sediment (A3-D3 to -D7) (see Table S2 in the supplemental material). Likewise, bacterial SSU rRNA genes from the genus *Rhodanobacter* were quantified in these sediments. The abundance of these genes varied from below detection (below 1.2E+03 copies/g, but generally much lower) to 9.96E+06 copies per gram of sediment, while the abundance of these genes as a proportion of the total bacteria SSU rRNA gene abundance varied from below detection (variable, but substantially below 4%) to 80.9% (see Table S2 in the supplemental material). *Rhodanobacter* SSU rRNA genes were generally low in relative abundance, with the exception of the sediment sample A3-D3, at a depth of 5.77 to 5.95 mbs in the Area 3 sediment borehole. At this acidic depth (pH = 3.2), *Rhodanobacter* bacteria dominated, as was seen in many of the acidic groundwater samples. Below this depth, the absolute and relative abundance of *Rhodanobacter* bacteria dropped significantly. In the circumneutral sediments at the top of the Area 3 borehole, *Rhodanobacter* SSU rRNA genes were detected at their greatest abundance (1.94 to 9.96E+06 copies per gram of sediment), though these sequences represented only a minor (<2.2%) component of the total bacterial community. Pyrotag sequencing analyses of bacterial SSU rRNA genes were also performed to characterize the structure and phylogenetic affiliation of the sediment microbial community and to determine the relative abundance of *Rhodanobacter* bacteria using a second molecular approach. The relative abundance of *Rhodanobacter* sequences within the pyrotag sequence libraries was consistent with the qPCR estimates, as observed for the groundwater data set (see Table S2 in the supplemental material).

The pyrosequence data were used to generate a UniFrac-based principal coordinate analysis (PCoA), which demonstrated that the bacterial community structure in the subsurface sediment samples was strongly impacted by interaction with contaminant chemistry (Fig. 4). The samples that were moderately impacted by

contaminants (Area 2 samples and A3-D1 and A3-D2 samples) had pH values greater than 5 and were separated largely on axis 1 from the heavily impacted sediments from Area 3 (A3-D3 to A3-D7) (Fig. 4A). Sediment samples from depths below 7 m in the Area 3 sediment core clustered strongly together, despite the lowermost sample having a circumneutral pH. The low-nitrate, low-pH sample (A3-D3) was separated from the other contaminant plume-impacted sediment samples primarily on axis 2 and from the elevated pH sediments on axis 1 as well, and the sequence library from this sample was largely composed of sequences from bacteria of the genus *Rhodanobacter* (Fig. 4B).

## DISCUSSION

**Impact of contaminant chemistry on overall bacteria and denitrifying bacteria in the subsurface of a nuclear legacy waste site.** Subsurface ecosystems have been termed "extreme" microbial habitats (26), and these results add to a small but growing database that supports this contention in the subsurface of U.S. DOE sites. The contaminant source zone (Area 3) of the ORIFRC site contains low microbial abundance and diversity where subsurface microbial communities are adapted to extremely high contaminant concentrations (low pH, high nitrate, heavy metals, and organic contaminants). Within the source zone of contamination, pH values are generally below 4, nitrate concentrations are generally above 10 mM and can exceed 100 mM, and uranium is among the many heavy metals present. In addition, the corrosive nature of the contaminant plume results in the dissolution of aluminum, with aluminum levels exceeding 600 mg/liter (~22 mM) in some areas (52). In the subsurface close to the source zone, bacterial abundance was previously shown to be exceedingly low, on the order of $10^2$ to $10^3$ cells/ml and below $10^4$ cells/g in the groundwater and sediments, respectively (12, 52). These prior data are consistent with the bacterial levels in the contaminated sediment (~$10^4$ SSU rRNA gene copies/g sediment) measured in this study. Somewhat higher levels of bacteria were detected in the acidic ($pH < 4$) groundwater in this study relative to the results of prior studies from the site (generally $10^4$ to $10^6$ SSU rRNA gene copies/ml groundwater) (12, 52).

Here, we demonstrate that bacteria from the genus *Rhodanobacter* were by far the most abundant microorganisms detected in the acidic, contaminated subsurface of a nuclear legacy waste site and that pH was the strongest determinant of the relative abundance of *Rhodanobacter* bacteria in the groundwater. The results were compiled from qPCR, fingerprinting with TRFLP, and pyrosequencing of rRNA gene amplicons, and as a result, this study provides one of the few environmental data sets where three such approaches for assessing the microbial community were applied over a large scale. Furthermore, by employing RNA-based analyses, we also provide evidence that the abundant *Rhodanobacter* sequences represent viable, active organisms and that *Rhodanobacter* bacteria dominate in the native (untreated) groundwater under the harshest subsurface conditions—elevated nitrate and uranium and low pH.

We had initially hypothesized that conditions in the ORIFRC site subsurface would provide selective pressure favoring denitrifiers and soil microorganisms that tolerate an acidic and often nutrient-starved environment. In pristine soils and sediments, nitrate concentrations are typically too low to select for large populations of denitrifying organisms, and denitrifiers are thought to rely on aerobic heterotrophy rather than on their denitrification capacity (48). At the ORIFRC site, the elevated levels of nitrate, coupled with low and variable oxygen levels, favor denitrifiers. Denitrifying bacteria have been detected and isolated from the ORIFRC subsurface (23, 25, 47), but the detection of ORIFRC site denitrifying bacteria, such as *Rhodanobacter* spp., through functional gene assays has been limited by the highly divergent sequences of denitrification genes from different microbial lineages (25). This sequence divergence limits the utility of general or universal functional gene primers for performing systematic surveys. Although *Rhodanobacter* isolates from the site are capable of complete denitrification (25, 42) and other studies have indicated the denitrification activity of *Rhodanobacter* bacteria (49), nitrate does not appear to be the major driving factor for the distribution of these organisms *in situ*. It is difficult to definitively ascertain, however, because of the autocorrelation of pH, U, and nitrate contamination. Additionally, nitrate levels in the acidic source zone are elevated and vary over roughly two orders of magnitude (from 1 to 100 mM), and they exceed the electron donor availability. Furthermore, low but detectable and variable levels of oxygen (often between 0.25 and 1 mg/liter in many acidic wells in the near-source zone) are present in most groundwater samples. Thus, while denitrification capabilities may be useful to subsurface bacteria during periodic anoxia, the frequent presence of low levels of oxygen (for example, in Area 3 well FW106, dissolved oxygen levels varied from 0 to 0.7 mg/liter over 6 years of monitoring) may confound the ability to track nitrate as a key ecological determinant at the site.

**Ecological niche of the genus *Rhodanobacter*.** The available data provide some hints as to the physiological properties of *Rhodanobacter* bacteria that can explain the extraordinary absolute and relative abundance of these organisms within contaminated and acidic groundwater and at least certain regions of the sediment. Ten species of the genus, including *R. denitrificans*, isolated from the ORIFRC site have been validly described and have been mainly isolated from soil (4, 10, 18, 29, 32, 40, 42, 50, 53, 56). Multiple members of the genus from subsurface materials from the ORIFRC site have been isolated, and most of these isolates are capable of growth at pH levels below 4 (data not shown) and of complete denitrification (25, 42). In corroboration with the results from this study, previous studies have shown that members of the genus grow under acidic conditions (4, 7, 49, 53), a clear physiological advantage in the ORIFRC site subsurface. Of the described members of the genus *Rhodanobacter*, only the organism most similar to *R. denitrificans* by SSU rRNA gene sequence (*Rhodanobacter thiooxydans*) is also capable of denitrification (32). Although the initial description of *R. thiooxydans* described only nitrate-to-nitrite reduction, we and others have verified its complete denitrification capability (42, 49). These data indicate that the capacity to conduct denitrification is restricted to two closely related species within the genus. *R. denitrificans* strain 2APBS1 and the closely related *R. denitrificans* strain 116-2 (isolated from Area 3 groundwater) have significant metabolic flexibility and can grow on a variety of different organic substrates, including excellent growth on acetate, a rare feature of the genus (42). Acetate is present at variable concentrations (occasionally upward of 1 to 2 mM) in the groundwater in the near-source zone at the ORIFRC site because it was used to stimulate denitrification and neutralize the residual liquid waste present in the former S-3 ponds during the 1980s as part of the shutdown of the ponds prior to capping (41). *R. denitrificans* strain 2APBS1 also has genes en-

coding enzymes needed for acid tolerance, including an arginine/ornithine decarboxylase, $Na^+/H^+$ antiporters, and organic acid catabolic enzymes (Jennifer Reed, personal communication). A close inspection of *R. denitrificans* strain 2APBS1 denitrification genes reveals that the organism contains two different *nirK* genes (25), and both of these are transcribed during growth under denitrifying conditions (data not shown). One of the genes is most similar to genes present in bacteria from betaproteobacterial lineages, though genome-genome comparison reveals that these genes are not syntenic (see Fig. S3 in the supplemental material). Conversely, the nitrous oxide reductase (*nosZ*) gene and associated genes from the reductase complex are syntenous with those of closely related gammaproteobacteria (see Fig. S4 in the supplemental material). These data suggest that *R. denitrificans* strain 2APBS1 has acquired its full denitrification capabilities in part via lateral gene transfer events, consistent with similar findings for other genes from the partial *Rhodanobacter* genome assembled from the FW106 groundwater metagenome (27). Further testing of this hypothesis will require genome sequencing of additional *Rhodanobacter* species, including *R. thiooxydans.*

Our results indicate that low-pH tolerance is a key physiological capability of the *Rhodanobacter* lineage that accounts for its success in the subsurface at the ORIFRC site. *Rhodanobacter* species are detected elsewhere in the site, even when pH is circumneutral or in noncontaminated areas, indicating their ubiquitous presence though not at elevated abundance in the subsurface. Decades of disposal of waste derived from uranium processing has led to acidic subsurface conditions that favor bacteria from the genus *Rhodanobacter*. Additionally, these conditions may have resulted in a dramatic die-off of other native soil bacteria, leading to conditions favoring lateral gene transfer (27, 38). In the subsurface sediment from Area 3, the shallowest depth has a level of bacterial SSU rRNA gene sequence diversity consistent with that found in pristine soils (45; data not shown). Below a depth of 5 m, where the sediment interacts with the contaminant plume, bacterial diversity is drastically diminished and bacterial rRNA gene abundance drops by nearly 4 orders of magnitude. At the ORIFRC site, the groundwater close to the source zone is only intermittently anoxic. These periods of anoxia in the nitrate-rich subsurface, however, favor bacteria capable of denitrification. Martinez et al. (38) suggested that metal sensitivity of native bacteria in the ORIFRC site subsurface likely facilitated the lateral gene transfer of metal resistance genes. Similarly, *Rhodanobacter* spp. may have acquired full denitrification capabilities via lateral gene transfer which, together with native acid tolerance, provided phenotypic features compatible with life in the contaminated subsurface. Although the growth rate for these organisms is slow (25), slow growth under conditions that select against natural competitors is a viable biological strategy. Studies from other ecosystems show that bacteria from the genus *Rhodanobacter* and other closely related genera (e.g., *Dyella*), appear to be able to, on occasion, dominate bacterial communities. For example, Campbell et al. (11) found that bacteria from the *Dyella-Rhodanobacter* lineages were abundant in acidic tundra soils that were experimentally manipulated with long-term nutrient fertilization. Elsewhere, van den Heuvel et al. (49) observed a high abundance of *Rhodanobacter* spp. in a denitrifying soil bioreactor operated long-term at pH 4. Together with this study, these data suggest a specific niche for *Rhodanobacter*—soils and groundwater at low pH with elevated nitrate. When various experimental manipulations of the subsurface at the ORIFRC site have raised the pH, removed nitrate, and/or provided substantial electron donor material (generally ethanol) in order to stimulate reductive immobilization of uranium, the measured abundance of *Rhodanobacter* bacteria in source zone wells has been very low (12, 13, 28).

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