Subsurface sediments were recovered from a 52-m-deep borehole cored in the 300 Area of the Hanford Site in southeastern Washington State to assess the potential for biogeochemical transformation of radionuclide contaminants. Microbial analyses were made on 17 sediment samples traversing multiple geological units: the oxic coarse-grained Hanford formation (9 to 17.4 m), the oxic fine-grained upper Ringold formation (17.7 to 18.1 m), and the reduced Ringold formation (18.3 to 52 m). Microbial biomass (measured as phospholipid fatty acids) ranged from 7 to 974 pmols per g in discrete samples, with the highest numbers found in the Hanford formation. On average, strata below 17.4 m had 13-fold less biomass than those from shallower strata. The nosZ gene that encodes nitrous oxide reductase (measured by quantitative real-time PCR) had an abundance of 5 to 17% relative to that of total 16S rRNA genes below 18.3 m and <5% above 18.1 m. Most nosZ sequences were affiliated with Ochrobactrum anthropi (97% sequence similarity) or had a nearest neighbor of Achromobacter xylosoxidans (90% similarity). Passive multilevel sampling of groundwater geochemistry demonstrated a redox gradient in the 1.5-m region between the Hanford-Ringold formation contact and the Ringold oxic-anoxic interface. Within this zone, copies of the dsrA gene and Geobacillus were the highest general abundance. The majority of dsrA genes detected near the interface were related to Desulfotomaculum spp. These analyses indicate that the region just below the contact between the Hanford and Ringold formations is a zone of active biogeochemical redox cycling.

The Hanford Site is located within the semiarid Pasco Basin of the Columbia Plateau in southeastern Washington State. The site contains radioactive waste stemming from years of nuclear weapon production. Large volumes of process wastewater were introduced into the 300 Area process ponds and infiltrated through the 4- to 10-m-thick vadose zone beneath the disposal facilities. Even after excavation of the bulk of contaminated sediments from the ponds, a groundwater uranium (U) plume has remained beneath the 300 Area, with the core of the plume exceeding the drinking water standard (30 μg/liter) (11, 41). The Columbia River forms the north and east boundaries of the Site. Upstream, the river is dam controlled; the river stage at the site can vary up to 3 m seasonally (6), and this variation causes near-shore water table fluctuations. These, in turn, affect dissolved U concentrations and distribution within the contaminant plume. Near the river, field-scale elements, such as aquifer sediments, groundwater, and the Columbia River, comprise a complex and dynamic microbial environment. The construction of experimental field sites, such as the Hanford Integrated Field Research Challenge (IFRC) site, in the 300 Area thus represent a unique and valuable natural laboratory for understanding the hydrologic, mass transport, and biogeochemical processes controlling contaminant fate and transport in the subsurface environment.

The role of subsurface microorganisms in transforming contaminants has not been a consideration in past decisions regarding environmental management of the Hanford Site. Microbial reactions can modify contaminant solubility, result in the precipitation or dissolution of mineral phases, and consume electron donors and reduce electron acceptors (and thereby alter the chemical and biogeochemical reactivity of microsites). Selected subsurface microbiology investigations were performed at Hanford, mainly in the western central plateau, in the early 1990s (15, 25, 34). These investigations largely predated the application of molecular ecology, and there has been only a small amount of recent work at Hanford related to microbial ecology (12, 14, 16, 19, 36). As a result, the significance of microbial community composition and functional potential for contaminant transformation in near-shore regions along the Columbia River corridor remains unknown.

For this initial study of subsurface sediments in the 300 Area of the Hanford Site, we characterized the vertical distribution of microbial biomass and community potential for relevant biogeochemical activities (reductions of sulfate, metals, and nitrate) across multiple geological formations, as well as groundwater geochemistry. We employed real-time quantitative PCR (qPCR) to assay the distribution of functional groups and constructed clone libraries for samples from selected depths to phylogenetically characterize the organisms. These measurements of the distribution of microbial biomass and functional bacterial groups were related to depth-dependent geochemical gradients in the aquifer determined using a passive multilevel sampler.

**MATERIALS AND METHODS**

**Site description and sampling.** Sampling took place in an experimental well field (Hanford Integrated Field Research Challenge [IFRC] site; [http://ifchanford.pnl.gov/](http://ifchanford.pnl.gov/)) in the 300 Area of the Hanford Site near Richland, WA. The site is about 250 m from the Columbia River. A deep characterization borehole (well number C6209), penetrating 52 m below the...
ground surface (bgs), was drilled during late July 2008 (6). Seventeen samples representing <1-m stratigraphic intervals were subjected to intensive microbiological analysis. These included three coarse-grained sediments from the saturated zone in the Hanford formation (9 to 17.4 m bgs) and six samples from the transition zone between the Hanford-Ringold contact (17.4 m bgs) and the fine-grained upper Ringold formation (18.9 m bgs). This transition zone comprises an aquifer that separates the upper aquifer from deeper Ringold formation sediments. Eight (30.8 to 51.5 m bgs) samples were analyzed from the Ringold formation’s unit E and lower mud strata and down to the Columbia River basalt flow top. Sampling methods and sediment lithology have been described elsewhere (6). The subsamples of cores were immediately stored in a −80°C freezer until processed for extraction of either DNA or phospholipid fatty acids.

Chemical measurements for groundwater and sediment samples. Groundwater samples were taken by equilibrating multilevel sampler (MLS) cells (53) in wells C6204 and C6209 at the IFRC site (http://ifichanford.pnl.gov/pdfs/18340.pdf) for more than 2 weeks prior to recovery for chemical measurements. These two wells are approximately 60 m apart, with C6204 near the southwest corner of the IFRC site well field and C6209 to the northeast, closer to the Columbia River. Due to the irregular subsurface topography, samplers deployed at the same depths in the two wells could access strata with disparate mineralogy or lithic texture. However, the profiles do allow comparisons of geochemistry across comparable units. Well C6204 had a 4-inch PVC (polyvinyl chloride) wire-wrap screen from 9.6 to 17.2 m below ground surface, corresponding to the geological strata from the water table into the upper reduced Ringold formation. Well C6209 was screened in the reduced Ringold formation from 18.9 m to 37.2 m.

Dissolved cations were determined on 0.2-μm-filtered, nitric acid-preserved samples using a Perkin Elmer Optima 2100 DV inductively coupled plasma optical emission spectrophotometer (ICP-OES). The ICP-OES was calibrated using ICP standards (Ultra Scientific, Inc., Kingsport, RI) in a dilution range of 0.5 to 3,000 μg liter⁻¹.

Dissolved anions were determined in 0.2-μm-filtered, un preserved samples using a Dionex ICS-2000 anion chromatograph, with an AS40 auto sampler. The analysis was performed using isocratic 15-min elutions with 23 mM KOH eluent at 30°C at a flow rate of 1 ml min⁻¹. A guard column (IonPac AG18 guard, Dionex catalog number 060551) and an analytical column (IonPac AS18, Dionex catalog number 060549) were column (IonPac AG18 guard, Dionex catalog number 060551) and an ionization detector (FID) at 295°C with an 80/100-mesh HayeSep A solid.

Dissolved sulfide was determined on unfiltered samples using the colorimetric methylene blue method (method 8131, Hach Co., Loveland, CO).

Dissolved gases were measured by gas chromatography at Oak Ridge National Laboratory using methods described in Spalding and Watson (44).

For gas analysis of H₂ and CH₄ in our laboratory, samples were run on a Trace Analytical RGA5 process gas analyzer (Peak Laboratories, LLC, Mountain View, CA). One-milliliter sample volumes were injected from the diffusion gas syringes to fill the 100-μl fixed injection loop. The calibration range for H₂ and CH₄ was 1 to 300 ppm. H₂ was run with a reductive gas detector (RGD) set at 265°C using a dual column consisting of a Supelco phase 700-059, 60/80-mesh, molecular sieve 5A, 31-μm by 1/8-in. stainless steel column and a Supelco phase 700-028, 60/80-mesh, Unibead, 31-μm by 1/8-in. stainless steel column set at 105°C. The carrier gas for the RGD was 99.9999% ultrapure nitrogen (product number X34380; Oxarc) with a nitrogen prepurifier (product number NP2; VICI Valco Instruments, Inc.) at 62 lb/ft² and a flow rate of 22 cm³ min⁻¹. The retention time for hydrogen was 35 s. Methane was run using a flame ionization detector (FID) at 295°C with an 80/100-mesh HayeSep A solid.

For the FID were ultrapure 99.9999% hydrogen (product number 33505; Oxarc) at 42 lb/in² and 25 cm³ min⁻¹ and ultrapure 99.9999% compressed air (zero air, product number X32070; Oxarc) at 66 lb/ft² and 34 cm³ min⁻¹. The retention time for methane was 61 s.

Organic carbon in sediment was analyzed in three subsamples from each of the following strata: Hanford formation (9.1 to 9.6 m bgs), oxidized Ringold formation (17.7 to 18.0 m bgs), and reduced Ringold formation (18.6 to 18.9 m bgs). Total Fe was only analyzed in the Hanford formation sample. The samples were sieved through a 4.75-mm mesh, and organic C or total Fe was analyzed at Huffman Laboratories, Inc. (Golden, CO), according to ASTMD standard methods (http://www.astm.org/MDIGITAL_LIBRARY/index.shtml). In addition, the concentrations of Fe(II) were analyzed by ferrozine assay (46) in the sieved Hanford formation sediments after extraction with 0.5 N HCl. Fifteen milliliters of anoxic 0.5 N HCl was added to the weighed sediments in an anaerobic glove bag (5% H₂ and 95% N₂; Coy Laboratory Products, Inc., Grass Lake, MI), and the samples were incubated at 100 rpm and 30°C for 24 h. The extraction was performed in triplicate.

**PLFA analysis.** Subsamples frozen at −80°C were shipped overnight to Microbial Insights, Inc., for analysis of phospholipid fatty acids (PLFA). Phospholipid biomarker analysis was performed as previously described (40). Briefly, PLFA were extracted with a single-phase chloroformalmethanol buffer system (54). The total extractable lipids were then separated, using a silicic acid column, into three lipid categories: neutral lipids, glycolipids, and polar lipids (18). After fractionation, the polar lipid portion was transesterified to form fatty acid methyl esters (FAMEs) by mild alkaline methanolsysis to release plasmalogens ethers as dimethyl acetals (18). Quantification and identification of individual PLFA were done using a combination of gas chromatography and mass spectroscopy.

**MPN determinations.** The medium for most probable number (MPN) culture was a mineral salts medium that mimicked the ionic strength and composition of groundwater augmented with a mix of organic substrates and a specific terminal electron acceptor. The mineral salts medium (pH 7.8) contained NaHCO₃ (1.1 mM), Ca(NO₃)₂ · 4H₂O (0.18 mM), CaCl₂ · 2H₂O (0.42 mM), MgSO₄ (0.21 mM), Na₂SO₄ (0.14 mM), KHCO₃ (0.07 mM), NH₄Cl (1 mM), KCl (1.34 mM), NaH₂PO₄ (0.43 mM), and HEPES (3 mM). The medium was supplemented with 10 ml liter⁻¹ of 10X Wolfe’s vitamin solution (3), 10 ml liter⁻¹ of 10X Wolfe’s mineral solution (3), and 10 ml liter⁻¹ electron donor mix (13 compounds at 40 mM each substrate: fructose, acetate, propionate, caprylic acid, methanol, ethanol, n-propanol, n-butanol, pyruvate, N-malate, succinate, N-lactate, and glycerol plus Casamino Acids [2.5 g liter⁻¹]). For fermenters, glucose was added (final concentration, 0.2 mM). After autoclaving, 1 ml of 1 g liter⁻¹ selenium and 0.1 ml of 100 mM Fe-nitrotriacetate were added.

Different electron acceptors were used to assess the cultivability of physiological groups. (i) For aerobic microorganisms, anoxic medium was used. All other tests entailed anaerobic incubations with the following specific modifications to the culture medium: (ii) fermenters, 0.2 mM glucose; (iii) denitrifiers, 5 mM NaNO₃; (iv) Mn-reducing bacteria, 5 mM MnO₂ and no HEPES buffer; (v) iron-reducing bacteria, 5 mM ammonium ferrihydrite; and (vi) sulfate reducers, 5 mM Na₂SO₄. To prepare MPN series, sediment slurries containing 1 g fresh sediment and 9 ml mineral salts solution were shaken at 200 rpm for 1 h at room temperature. The original sediment slurry was then serially diluted by adding 1 ml of each dilution to the 9 ml of mineral salts solution to a final sediment mass fraction of 10⁻⁶. Twenty microliters of each dilution was used to inoculate 180 μl of medium in the wells of microtiter plates (Corning, New York, NY), with 8 replicates per dilution. After incubation, the plates were covered with sterile lids (corner notch lid; Corning, New York, NY) and wrapped with Parafilm to avoid water loss by evaporation. All MPN series (except the aerobes) were incubated for 12 to 24 weeks at room temperature in an anaerobic chamber. Nitrate reducing medium was checked under the stereoscope for the growth of denitrifiers. Precipitation or clearing in SO₄²⁻, Fe⁺³, and Mn⁴⁺ reducing media was
visually examined under a stereoscope. The media for aerobes and fermenters were inoculated onto a solid medium and tested for growth: PYT80 (80 mg of each peptone, yeast extract, and tryptone) was used for aerobes, and PYT80 plus 5 mM glucose for anaerobes. Phytagel (Sigma-Aldrich, St. Louis, MO) at 0.8% plus 6.3 mM MgSO4 was used as the gelling agent.

**DNA extractions, cloning, and preparation of plasmid standards.** DNA was extracted from 10 to 20 g of sediment from each stratum. Briefly, a prelysis washing step was applied by mixing about 10 g of soil with 25 ml of phosphate buffer 1 (pH 6.6) and 1.5 ml of 100 mM Al2(SO4)3 solution and then adjusting the pH to 8 for centrifugation (20). A lysis buffer containing 2 ml of SDS lysis mixture (100 mM NaCl, 500 mM Tris [pH 8], 10% [wt/vol] SDS) and 15 ml phosphate buffer (pH 8.0) was added to each sediment pellet, followed by three cycles of freeze-thaw cell lysis at −80°C for 20 min and 65°C for 5 min per cycle. The final lysis step was done by incubating the samples at 65°C for 30 min, and then they were centrifuged at 5,000 × g for 2 min. The supernatant containing genomic DNA was collected and precipitated by mixing with 0.1 volume of 5 M NaCl and 1.0 volume of isopropanol. The extracted DNA, as well as other coextracts (e.g., protein), was centrifuged at 8,500 × g for 60 min at room temperature. The pellet was cleaned up by extraction using an equal volume of phenol/chloroform/isoamylalcohol (25:24:1), followed by two extractions with chloroform/isoamylalcohol (24:1). The genomic DNA was further purified with a Montage PCR kit (Millipore Corp., Bedford, MA) by washing three times with 450 μl of 10 M Tris.

Isolation of genomic DNA from cultured strains (as standards for qPCR) was done using a MoBio PowerSoil DNA isolation kit according to the manufacturer’s manual. For targeting 16S rRNA genes, plasmid clones containing 16S rRNA gene fragments were chosen as standards for qPCR assays. DNA fragments containing the small subunit (SSU) rRNA gene were amplified by PCR with genomic DNA of reference strains or environmental samples listed in Table 1. The bacteria-specific primers 27F and 1492R were used to amplify 16S rRNA genes of Shewanella oneidensis MR-1, Anaeromycobacter dehalogenans strain K, and Geobacter sulfurreduens. The dsrA and nosZ gene fragments were amplified from Desulfovibrio desulfuricans strain P28-1 using primers DSR-1F and DSR-4R, and environmental DNA (from the 9.5-m depth) using nosZ-2F and nosZ-2R.

To generate the above-named gene fragments, 50-μl PCR mixtures contained 1× Easy-A high fidelity master mix (Stratagene Ltd.), 100 to 1,000 nM primers (Table 1), and 1 μl template DNA. PCR was performed in a PTC-225 Peltier thermal cycler (MJ Research) with an initial denaturation step of 95°C for 3 min, followed by 25 cycles of 95°C for 30 s, annealing at temperatures referenced in the above table for 30 s, and 72°C for 1.5 min, with a final extension at 72°C for 10 min. PCR products were purified with a MinElute PCR purification kit (Qiagen Inc., Valencia, CA) according to the manufacturer’s instructions. The amplification products were visualized by electrophoresis through a 1.0% agarose gel in 1× TAE (40 mM Tris-acetate, 1 mM EDTA) containing eudiithm bromide (0.50 mg ml⁻¹). The amplicons were cloned using the TOPO TA cloning kit (Invitrogen, Carlsbad, CA) and served as standard templates. Plasmids used as standards for qPCR assays were purified using a PureLink quick plasmid miniprep kit (Invitrogen) according to the manufacturer’s protocol and sequenced to confirm the insertions. Linearized plasmids were produced by digestion with the restriction endonuclease EcoRI (Invitrogen, Carlsbad, CA) according to the manufacturer’s protocol and purified using a MinElute PCR purification kit (Qiagen, Inc., CA). The DNA concentrations of purified plasmids were determined fluorometrically using a Picogreen DNA quantification kit (Invitrogen) and a Turner Designs TD-700 fluorometer according to the manufacturer’s manual.

**Quantitative real-time PCR assays.** All reactions were performed with a StepOnePlus real-time PCR system (Applied Biosystems, Inc., Foster City, CA) programmed for 45 cycles according to the references listed in Table 1. Power SYBR green PCR master mix was used for all SYBR green-based assays. For the GEO494F/825R primer pair, optimal primer concentrations were determined using a matrix of concentrations that ranged from 100 to 1,000 nM forward and reverse primers. The primer combination with the lowest cycle threshold (Ct) value (number of PCR cycles that elapse before the threshold is reached) and the highest ΔRn value (baseline-subtracted fluorescent reading normalized to the reference dye) were selected. For all SYBR green-based assays, the melting curves of amplicons were analyzed to ensure that a single homogeneous product was generated.

Linearized plasmid DNA with inserts of specific gene fragments was used to establish standard curves that were included in each run. The standard contained different quantities of cloned gene fragments, spanning 8 orders of magnitude from 10¹ to 10⁸ gene copies per PCR well. To minimize the effects of inhibitors in assays, environmental DNA was diluted to 5 to 10% of the original concentrations, and duplicate wells each containing 2.5 μl of diluted DNA were run for each sample. The results are reported as the relative abundance of gene copies, normalized to the total bacterial and archaeal 16S rRNA gene copy numbers as reported in Lin et al. (28).

**Constructing clone libraries for nosZ, dsrA, and Shewanella 16S rRNA genes.** Two clone libraries of nosZ genes were constructed from qPCR products using DNA samples from depths of 9.5 m (Hanford formation) and 18.9 m (just below the Ringold oxic-reduced interface), re-

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**Table 1** Primers, probes, reference strains, and clones used in the study

<table>
<thead>
<tr>
<th>PCR Type</th>
<th>Target</th>
<th>Source of gene fragments inserted into plasmid standards</th>
<th>Primer (conc [μM])</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>End-point PCR</strong></td>
<td>dsrAB</td>
<td>she211f (0.4)</td>
<td>DSR-1F (0.4)</td>
</tr>
<tr>
<td>Shewanella spp.</td>
<td></td>
<td>She1259r (0.4)</td>
<td>DSR-4R (0.4)</td>
</tr>
<tr>
<td><strong>Real-time PCR</strong></td>
<td>Domain Bacteria</td>
<td>Shewanella oneidensis MR-1</td>
<td>331F (0.1)</td>
</tr>
<tr>
<td></td>
<td>Geobacteriae</td>
<td>Methanospirillum hungatei</td>
<td>797R (0.1)</td>
</tr>
<tr>
<td></td>
<td>spp.</td>
<td>Anaeromycobacter dehalogenans strain K</td>
<td>ARCH349F (0.8)</td>
</tr>
<tr>
<td></td>
<td>Geobacter spp.</td>
<td>Geobacter sulfurreduens</td>
<td>ARCH806R (0.8)</td>
</tr>
<tr>
<td></td>
<td>Shewanella spp.</td>
<td>Shewanella oneidensis MR-1</td>
<td>60F (0.4)</td>
</tr>
<tr>
<td></td>
<td>dsrA (dissimilatory sulfite reductase gene)</td>
<td>Desulfovibrio desulfuricans</td>
<td>461R (0.4)</td>
</tr>
<tr>
<td></td>
<td>nosZ (nitrous oxide reductase gene)</td>
<td>nosZ gene of Ochrobactrum anthrophi from sample depth 9.5 m</td>
<td>GEO494F (0.15)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>GEO825R (0.15)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>She112f (0.3)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>She220r (0.3)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>DSR-1F (0.4)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>DSR-4R (0.4)</td>
</tr>
<tr>
<td><strong>Primers</strong></td>
<td></td>
<td></td>
<td>nosZ-2F (1.0)</td>
</tr>
</tbody>
</table>

Reference(s)Forward Reverse Probe Reference(s)
respectively. Clone libraries of dsrA gene fragments were constructed by amplifying DNA from depths of 17.4 m and 18.3 m with primer pair DSR-1F and DSR-4R (Table 1). Thirty-six white colonies from each nosZ and dsrA library and 60 colonies from the Shewanella 16S rRNA gene clone library were picked for plasmid isolation and sequencing. Shewanella 16S rRNA genes were amplified with primers She211f and She1295r (Table 1) from samples at depths of 9.5 m, 18.9 m, and 47.3 m. Amplicons from these three depths were purified and quantified. Equal amounts of amplicons from each sample were pooled for ligation and transformation. Forty-eight white colonies were picked to prepare glycerol stocks. The PCR conditions for amplification of each gene target have been described above. Glycerol stocks, after a 12-hr incubation, were sent to Agencourt Bioscience Corporation for plasmid isolation and one-way sequencing (<800 bp per read).

Phylogenetic analysis of gene sequences. The nosZ and dsrA gene sequences were translated in NCBI’s open reading frame finder (http://www.ncbi.nlm.nih.gov/projects/gorf/), and their phylogenetic affiliations checked using the blastp program. Sequences confirmed to be nosZ or dsrA genes were aligned in MEGA4 with functional gene sequences downloaded from Functional Gene Pipeline (http://fungene.cme.msu.edu/). Sequences that were more than 97% similar were clustered, and one representative sequence was used to construct a neighbor-joining tree. Those nearest neighbors and representative nosZ or dsrA sequences from the database were included in the tree construction.

Shewanella 16S rRNA gene sequences were imported into ARB (32) and aligned using FastAligner. The nearest neighbors were marked together with sequences in this study to generate a neighbor-joining tree with a bootstrap analysis. Sequences that were more than 99% similar were considered identical, and one representative sequence was kept for construction of a neighbor-joining tree.

Nucleotide sequence accession numbers. The above-described sequences have been assigned GenBank accession numbers HQ622631 to HQ622640 (nosZ gene), HQ622641 (Shewanella 16S rRNA gene), JN185617 to JN185621 (Pseudomonas 16S rRNA gene), and HQ622642 to HQ622645 (dsrA gene).

RESULTS

Geochemistry in the subsurface aquifers. Geochemical profiles were sampled with 10-cm resolution across the Hanford-to-Ringold transition zone in well C6204; the deepest samples corresponded to the upper fine-grained Ringold subunit. Steep chemical gradients occurred immediately below the Hanford-Ringold contact (Fig. 1). Dissolved O2 was near saturation with respect to the atmosphere in groundwater within the transmissive Hanford formation but decreased to below detection limits within 1 m below the contact. Concentrations of nitrate and sulfate were relatively constant above the Hanford-Ringold contact, averaging 385 ± 25 μM (mean ± standard deviation) and 469 ± 9 μM, respectively. Nitrate concentrations decreased below the contact and were undetectable below 19 m, whereas nitrite concentrations were undetectable above 16 m and peaked just below the Ringold oxic-anoxic interface in well C6204. Sulfate increased in concentration at about 15.5 m, just below the Hanford-Ringold contact in well C6204. MLS placement in well C6209 allowed analysis of deeper strata; the shallowest samplers were located below the oxidized zone and within the fine-grained Ringold. Nitrate and nitrite were undetectable in the strata of the Ringold formation in well C6209. Sulfate decreased from 250 μM just below the fine-grained Ringold to concentrations <100 μM below 20 m; sulfide was detected in all samples with maxima at 19 and 37.2 m. Dissolved Mn(II) and Fe(II) were below detection in the Hanford formation groundwater. Dissolved Fe(II) in well C6204 peaked at ~2.4 μM in the fine-grained Ringold, while the maximum dis-
The average PLFA concentration was 519 pmol PLFA g \(^{-1}\) (below 17.7 m) (Table 2). In three Hanford formation samples, Hanford formation (9.5 to 17.4 m) than in the Ringold formation respectively. Hanford formation, oxic Ringold formation, and upper reduced strata were 2-fold lower in the Hanford than in the Ringold formation, and they were irregularly detected in the Ringold sediments. Unfortunately, low total PLFA levels (and hence no detection of the stress markers) in some Ringold samples prevented a more comprehensive analysis of physiological stress in different geological units.

There was a 6-fold decline in cultivatable aerobes from the Hanford to the Ringold sediments (Table 2). On average, aerobes represented 1.3% ± 2.2% of total PLFA-based cell estimates. Both fermenters and denitrifiers were detected throughout the sampled profile but showed only minor variations, about 0.32% ± 0.33% and 0.41% ± 0.38% of the total PLFA biomass, respectively. Cultivable microorganisms capable of Fe(III) and Mn(IV) reduction represented about 0.01% of total biomass in the Hanford formation, and they were irregularly detected in the Ringold sediments. Note that despite the high dissolved oxygen concentrations in Hanford formation groundwater, sulfate reducers were detected in Hanford sediment samples. The MPN counts of sulfate reducers were 2-fold lower in the Hanford than in the Ringold formation (0.9 × 10^3 versus 1.8 × 10^3 cells g \(^{-1}\)).

### Relative abundances of functional bacterial groups

The relative abundance of both phylogenetic and functional genes was determined by normalizing to the absolute copy numbers of total bacterial plus archaeal 16S rRNA genes to factor out the 100-fold differences in total biomass between samples (Fig. 2). The nosZ gene, encoding nitrous oxide reductase that catalyzes the reduction of N\(_2\)O to N\(_2\) in microbial denitrification, was quite prevalent, with an abundance of 5 to 17% relative to total 16S gene copy numbers below 18.3 m and 0.5 to 4.7% in samples above 18.1 m (Fig. 2A). Sulfate-reducing potential was assayed by analysis of samples collected below 18.3 m.
dsrA gene copies and ranged from below the detection limit (2 × 10^2 gene copies g^-1) to 7% of total 16S rRNA genes in Ringold sediments; dsrA gene copies were near the detection limit in Hanford sediments (Fig. 2B). For the detection of metal-reducing bacteria, no functional gene primers are available, so phylogenetic primers for several taxa known to be important in this process were applied. Detection of Geobacteraceae, Anaeromyxobacter spp., or Shewanella spp. was sporadic, and their abundance was generally below 0.5% of total 16S gene copy numbers, with the exception that Geobacteraceae accounted for 4.2% and 1.7% at the depths of 17.4 m and 51.2 m, respectively (Fig. 2C).

Phylogenetic analysis of nosZ, dsrA, and Shewanella 16S rRNA genes. Because denitrifying bacteria were prevalent throughout the Hanford 300 Area subsurface sediments, we compared nosZ gene sequence diversity in clone libraries from two depths: 9.5 m (Hanford formation) and 18.3 m (Ringold formation). When aligned on a nosZ phylogenetic tree, three clusters were found (Fig. 3); there was one sequence (HF-B5) with only 50% sequence similarity to any in the current nosZ gene database. The most abundant sequences in libraries from both the Hanford and Ringold samples (48% of total sequences) were closely affiliated (>97% similarity) with nosZ genes from the alphaproteobacterium Ochrobactrum anthropi. Eighteen percent of sequences (all from the Ringold formation sample) had the closest association (90% similar-

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**TABLE 3** PLFA biomarkers of microbial physiological stress at different depths, measured in terms of the cyclopropyl/monoenoic precursor ratios and the trans/cis PLFA ratios

<table>
<thead>
<tr>
<th>Geological units</th>
<th>Depth (m)</th>
<th>Cyclopropyl/monoenoic precursor ratio cy17:0/16:1w7c</th>
<th>cy19:0/18:1w7c</th>
<th>Trans/cis PLFA ratio 16:1w7t/16:1w7c</th>
<th>18:1w7t/18:1w7c</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hanford formation</td>
<td>9.5</td>
<td>0.52</td>
<td>0.72</td>
<td>0.11</td>
<td>0.12</td>
</tr>
<tr>
<td></td>
<td>9.6</td>
<td>0.51</td>
<td>0.68</td>
<td>0.22</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>9.8</td>
<td>0.66</td>
<td>0.85</td>
<td>0.1</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>17.4</td>
<td>23.81</td>
<td>10.53</td>
<td>0.59</td>
<td></td>
</tr>
<tr>
<td>Ringgold formation</td>
<td>17.7</td>
<td></td>
<td>0.22</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>18.1</td>
<td></td>
<td>1.75</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>30.8</td>
<td>1.47</td>
<td>0.37</td>
<td>0.35</td>
<td></td>
</tr>
<tr>
<td></td>
<td>38.4</td>
<td></td>
<td>0.49</td>
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<td></td>
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**FIG 2** Relative abundances of phylogenetic and functional microbial groups, normalized to total Bacteria and Archaea 16S rRNA gene copy numbers. Upper and lower dotted lines represent the Hanford-Ringold contact and Ringold oxic-anoxic interface, respectively.

**FIG 3** Neighbor-joining tree of nosZ genes amplified from DNA samples at 9.5 m (circles) and 18.3 m (triangles). Bootstrap values larger than 50 are shown on each branch. Values in parentheses indicate the number of identical sequences in the libraries.
Hanford Site Subsurface Biomass and Anaerobic Respiration

The relative abundance of dsrA gene copy numbers underwent a dramatic change near the Ringold oxidized-reduced interface at 18.3 m, suggesting a potential shift in the community composition of sulfate-reducing bacteria. To compare diversity between oxidized and reduced sediments that have similar textural characteristics, dsrA gene clone libraries were constructed from oxic (17.4 m) and reduced (18.3 m) Ringold sediments. Two sequence clusters were identified (Fig. 4): one related (91% similarity) to Desulfotomaculum sp. and the other most closely affiliated (89% similarity) with an uncultured aquifer clone. Both the oxidized and reduced strata contained sequences affiliated with Desulfotomaculum thermosapovorans, a spore-forming, sulfate-reducing bacterium belonging to the order Clostridiales. Sequences affiliated with leachate-polluted aquifer clones were found only in the reduced Ringold sample.

We confirmed that the amplicons from Shewanella-specific primers were authentic by constructing a clone library after mixing PCR products from depths of 9.5 m, 18.9 m, and 47.3 m. Forty-one of 48 nonchimeric sequences of Shewanella had more than 99% sequence similarity to and affiliated with the cultivated strain Shewanella oneidensis MR-1. The other 5 sequences were found to represent another clade, a Pseudomonas sp. (data not shown).

DISCUSSION

Biomass distribution within the Hanford Site subsurface. The highest biomass levels were found in the highly transmissive Hanford formation. Our finding is consistent with data from other terrestrial subsurface environments, where microbial biomass and activity generally decline as a function of depth and then remain relatively constant over a range of deeper sediments (27). The subsurface in the Hanford 300 Area contains multiple geological units and, also, physical and chemical transition zones, such as the Hanford-Ringold contact and the oxidized-reduced interface in the fine-grained Ringold unit. These stratigraphic and lithologic features create heterogeneity in sediment properties, such as porosity and texture, between geological units and could therefore affect microbial biomass distribution (27). The dramatic decline in biomass from the Hanford to the Ringold formation was not correlated to organic carbon contents, which were similar in both strata. The high transmissivity in the Hanford aquifer compared to that in the fine-grained Ringold sediments likely provides a much greater advective flux of limiting nutrients to microbes, which could then theoretically sustain higher biomass content and levels of metabolic activities. The analysis of physiological stress markers in PLFA profiles is also consistent with the idea that microbes in the Ringold formation encounter lower nutrient fluxes than those in the Hanford formation.

Geochemical gradients and their relationship to microbial distributions. Groundwater chemical profiles indicated a sharp transition in redox conditions below the Hanford-Ringold formation contact. Oxygen and nitrate became depleted across the contact and within the fine-grained Ringold subunit, whereas reduced products of anaerobic respiration [nitrite, Fe(II), Mn(II), sulfide, and methane] exhibited the opposite trend, increasing with depth. This redox zonation likely reflects diverse microbial activities operating in the 300A subsurface that are also vertically stratified and responsible for driving the reactions that contribute to such stratification. We employed culture-independent and culture-dependent techniques to assay the potential for anaerobic respiratory processes, although we understand the difficulty in directly linking gene copy number and enrichment experiments to in situ microbial physiology and function (50).

The qPCR and MPN analyses indicated a broadly distributed potential for nitrate reduction throughout the subsurface strata. The geochemical profiles, however, suggest that microbial nitrate reduction would be restricted to the strata just below the Hanford-Ringold contact, where O2 becomes depleted and nitrate is detectable. The denitrifying microbial populations in the 300 Area subsurface sediment were dominated by Ochrobactrum anthropi and Achromobacter sp., based on nosZ gene libraries. There are culturing bacterial genera, such as Acidovorax, Acinetobacter, Alcaligenes, Hyphomicrobiurn, Ralstonia, Sphingomonas, etc. (1, 55). We only amplified nosZ genes in this study and cannot exclude the possibility that other N reductases and taxa are important components (17). The high abundance of O. anthropi in denitrifying populations has not been reported in other subsurface habitats. However, there is very poor understanding of how mineralogic and geochemical factors select for specific functional microbes at different sites. For example, the Hanford Ochrobactrum nosZ clones had 97% similarity with Ochrobactrum anthropi YD50.2, a novel denitrifying bacterium found to tolerate relatively high levels of reactive nitrogen species (NO and NO2-) (13). Although the bulk groundwater concentrations of nitrite were much lower than those reported by Doi et al. in a study of reactive nitrogen species tolerance (13), the physically heterogeneous subsurface sediments may structure the unique denitrifying populations in the Hanford Site subsurface. If the Hanford Ochrobactrum population possesses the high nitrite tolerance of O. anthropi YD50.2, this repre-
sents a strategy for selective cultivation of this numerically abundant subsurface microbe.

Overall, the relative abundance of metal- and sulfate-respiring bacteria was quite low in the 300A subsurface sediments. The low population levels suggested that the rates at which these processes were occurring were relatively low and that this could be a consequence of limited availability of appropriate electron donors and/or acceptors. However, genes or taxa associated with these processes were detected, and both field and laboratory studies have demonstrated that the abundances of metal and sulfate reducers can increase significantly when appropriate environmental and nutritional conditions are imposed (2, 8, 39, 48, 51).

It is noteworthy that functional groups of anaerobic respirers were detected (at low abundance) by molecular and cultivation approaches in several samples from the oxic Hanford formation. These sediments are physically and mineralogically heterogeneous and could contain microsites where advection forces are low (29) and hot spots (33) where electron donors are relatively concentrated. This circumstance would generate microenvironments of O2 depletion where anaerobic respiration could occur. The high proportion of Fe(II) within the total Fe content of Hanford formation sediments demonstrates that zones depleted in O2 are present and suggests that microbially reduction of Fe(III) might occur in the oxic Hanford formation sediment.

The maxima in abundance of dsrA genes and Geobactereaceae 16S rRNA genes below the Hanford-Ringold contact were consistent with the geochemical profiles, which indicated steep redox gradients and peaks in the metabolic products of Fe(III) and sulfate reduction. The zonation of oxidized and reduced chemical species is commonly encountered in saturated sediments in freshwater and marine habitats (5), where these processes are driven by the sedimentation of organic matter from the overlying water. The nature of the driving forces for biogeochemical reduction were less clear in this environment and will require extensive further study to adequately define. The concentration of dissolved organic carbon in groundwater has been assayed to be <25 μmol liter−1, and sedimentary organic C ranged from <42 μmol/g to 67 μmol/g in Hanford and upper Ringold sediments. Future work will consider the capacity for inorganic electron donors in driving biogeochemical reductions in the subsurface (9, 37).

The reason for the increase in sulfate concentration with depth is unclear but is likely due to a combination of oxidation of iron sulfide and leaching of sulfate from the reduced region of the fine-grained Ringold formation. Similar findings have been observed in other aquifers (9, 42), where confining beds contained higher concentrations of sulfate than the aquifer, as the developed concentration gradient allowed sulfate to diffuse. Pyritic S was detectable in reduced Ringold sediment from well C6209 cores but undetectable in the overlying oxidized Ringold material (unpublished observations). The decrease in dissolved O2 and nitrate across the fine-grained Ringold could be due, at least in part, to microbial oxidation of sediment-associated reduced S and/or Fe. Consistent with this hypothesis is the maintenance of a lithotrophic Fe(II)-oxidizing, nitrate-reducing enrichment culture by E. Shelbyolina (personal communication), in which Fe in reduced Ringold sediments is the major available electron donor.

Gas concentration profiles suggested an upward flux of H2 and CH4 from the deeper aquifer, and CH4 was detectable from the bottom of the screened interval in well C6209 to the base of the Hanford formation. This observation suggested the existence of hydrogen-consuming and CH4-producing microbial populations in the reduced Ringold formation (10, 45) and the potential for methanotrophy in both the oxic and anoxic Hanford subsurface.

The vertical arrangement of geological units and their distinct sediment textures make it likely that field-scale features (Hanford-Ringold contact, Ringold oxic-reduced interface, and silt barriers) in the Hanford Site subsurface exert a significant control on the transfer and flux of energy and material, hence limiting microbial biomass distribution through groundwater flow and river water infiltration. Additional system complexities that may impact temporal and spatial dynamics of the microbial community in the Hanford unconfined aquifer arise from the fluctuating hydrological gradient and the flux of reduced inorganic energy sources (e.g., H2 and CH4) from deeper strata. The facts that H2 and CH4 are detectable in the Hanford formation and that their concentrations are quite high in the Ringold formation suggest organic matter fermentation combined with electron acceptor limitation. Therefore, it would be interesting to determine the source(s) of H2 and CH4 in the system and the extent to which the upward fluxes of fermentation and methanogenesis products support microbial biomass and activity above the aquitard.

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