Effects of legacy nuclear waste on the compositional diversity and distributions of sulfate-reducing bacteria in a terrestrial subsurface aquifer

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Received 29 March 2005; revised 8 September 2005; accepted 8 September 2005.
First published online 19 December 2005.

doi:10.1111/j.1574-6941.2005.00039.x

Editor: Gary King

Keywords
sulfate-reducing bacteria; *dsrAB*; metals; community stability.

Abstract

The impact of legacy nuclear waste on the compositional diversity and distribution of sulfate-reducing bacteria in a heavily contaminated subsurface aquifer was examined. *dsrAB* clone libraries were constructed and restriction fragment length polymorphism (RFLP) analysis used to evaluate genetic variation between sampling wells. Principal component analysis identified nickel, nitrate, technetium, and organic carbon as the primary variables contributing to well-to-well geochemical variability, although comparative sequence analysis showed the sulfate-reducing bacteria community structure to be consistent throughout contaminated and uncontaminated regions of the aquifer. Only 3% of recovered *dsrAB* gene sequences showed apparent membership to the *Deltaproteobacteria*. The remainder of recovered sequences may represent novel, deep-branching lineages that, to our knowledge, do not presently contain any cultivated members; although corresponding phylotypes have recently been reported from several different marine ecosystems. These findings imply resiliency and adaptability of sulfate-reducing bacteria to extremes in environmental conditions, although the possibility for horizontal transfer of *dsrAB* is also discussed.

Introduction

Metal contamination of soil and groundwater is a persistent problem at hundreds of sites across the United States where uranium was mined or processed, or nuclear research was conducted (Riley & Zachara, 1992; Wolbarst *et al*., 1999). Engineered remedial actions (i.e., capping, soil excavation, groundwater pump and treat) are often necessary for containment and treatment of source zone areas that have high contaminant concentrations, but more passive, cost-effective technologies are of great interest for widespread peripheral areas that have lower contaminant concentrations. Metal-reducing bacteria are ubiquitous in the environment (Lovley, 1993b; Abdelouas *et al*., 2000; Senko *et al*., 2002) and are capable of affecting the solubility of redox metals (Lovley, 1993a; Nealson *et al*., 2002). Bioremediation has been proposed as an effective strategy for *in situ* precipitation of heavy metals and uranium in the subsurface (Lovley, 1995; Nealson *et al*., 2002).

The sulfate-reducing bacteria (SRB) have been studied extensively for their primary role in pollutant and metal geochemistry (Coates *et al*., 1996; Abdelouas *et al*., 2000, 1998; Spear *et al*., 2000). SRB reductively precipitate redox metals by the production of sulfide, although this may not be a significant pathway for U(VI), or through enzymatic pathways (Lovley *et al*., 1991; Lovley & Phillips, 1992b; Abdelouas *et al*., 1998). Enzymatic reduction of U(VI) has been demonstrated previously for *Desulfovibrio* spp. (Lovley & Phillips, 1992a; Payne *et al*., 2002), *Desulfuromonas acetoxidans* (Aubert *et al*., 1998) and *Desulfotomaculum reducens* (Tebo & Obraztsova, 1998). *Desulfovibrio desulfuricans* has been shown to readily reduce U(VI) at concentrations as high as 24 mM in the presence of 100 μM levels of contaminating ions (Mg^{2+}, Ca^{2+}, Fe^{2+}, Mn^{2+}, Co^{2+}), competing electron acceptors (SO_4^{2-}, NO_3^{+}) or toxic metals (Ni^{2+}, Cu^{2+}) in acidic and circumneutral mine drainage waters and uranium-contaminated groundwater from the Hanford, WA, USA DOE site (Lovley & Phillips, 1992a). The

increased abundance of *Desulfosporosinus* spp. 16S rRNA genes coincided with accelerated precipitation of U(IV) in enrichment cultures from the Midnite uranium mine (WA, USA) (Suzuki *et al*., 2003). Furthermore, putative *Desulfotomaculum dsrAB* sequences were the predominant genotype from groundwater collected from uranium mill tailings at the Shiprock UMTRA site (NM, USA) (Chang *et al*., 2001). SRB contribute to metal cycles and therefore may be central to remediation of metal- and radionuclide-contaminated environments.

The US Department of Energy's Natural and Accelerated Bioremediation Research Program (NABIR) established a Field Research Center (FRC) in Bear Creek Valley on the Oak Ridge Reservation (Oak Ridge, TN) to address the fate and transport of, and to identify remedial solutions for, nuclear legacy waste contamination in the subsurface environment. This heavily contaminated subsurface aquifer is an extreme environment. Metal-reducing bacteria, such as SRB, capable of sustained metabolism and cell growth during chronic exposure to extremes in environmental conditions may be useful bioremedial candidates for the immobilization of metals *in situ*. To date, the SRB have not been investigated in this subsurface system. The goal of this study was to determine the long-term effects of contamination on the diversity and distributions of SRB.

Materials and methods

Study site and groundwater sampling

Groundwater samples were collected from the FRC established by the DOE–NABIR program in the Bear Creek Valley near the former Y-12 plant on the Oak Ridge Reservation. During the 30-year history of nuclear production at this site, approximately 10 million liters of aqueous waste containing uranium, NO_3^- (from nitric acid), SO_4^{2-} (from sulfuric acid), organics, and heavy metals were discharged from Y-12 into four unlined settling basins (hereafter referred to as the S3 source ponds). Contaminant leaching from the source ponds and by groundwater transport has resulted in extensive subsurface contamination spanning 243 acres of the Bear Creek Valley (http://www.esd.ornl.gov/nabirfrc/).

Six FRC groundwater sampling wells were selected along the contaminant plume flow path for molecular investigation of the subsurface sulfate reducing community. Groundwater sampling wells were categorized into three groups based on contaminant burden, which was operationally defined by proximity to the source zone and groundwater chemistry profiles. Three high-contaminant burden sites (FW-010, FW-005, FW-015) were selected within the immediate perimeter (approximately 20 m) of the S3 source ponds, and all three sites were characterized by low groundwater pH and high inorganic contaminant concentrations. Two moderate-contaminant burden sites (FW-003, TPB-16) were located approximately 275 m away from the S3 source ponds and characterized by circumneutral pH groundwater and substantially lower inorganic contaminant concentrations. A zero-valent iron-based reactive barrier was installed at site TPB-16 in 1997 for passive groundwater remediation (12). A single unspoiled background site (FW-300) 2 km away from the source area was also examined. Two liters of groundwater were collected in sterile amber glass bottles from screened sampling wells in December 2000 and stored at 4 °C for no more than 4 days prior to DNA extraction. Groundwater chemistry was analyzed by standard procedures.

DNA extraction and *dsrAB* sequence analysis

Total cellular biomass was collected by centrifugation of groundwater. DNA extraction and purification (Zhou *et al*., 1996), *dsrAB* gene amplification, cloning, restriction fragment length polymorphism (RFLP) analysis, and sequencing followed standard procedures and have been described in detail elsewhere (Liu *et al*., 2003). For simplicity, sequence clades that were composed exclusively of *dsrAB* sequences from the FRC were collapsed and a randomly selected clone from each clade used for presentation. *dsrAB* sequences were assembled and edited in Sequencher™ (v. 4; Gene Codes Corporation, Ann Arbor, MI), aligned in ClustalW (Thompson *et al*., 1994), and bootstrapped (500 resamplings) evolutionary distance trees were constructed using the neighbor-joining method in MEGA version 2.1 (Kumar *et al*., 2001).

Statistical analyses

Principal component analysis (PCA) was performed using the SYSTAT statistical computing package (SYSTAT version 10.0; SPSS, Inc., Chicago, IL). PCA simultaneously considers many correlated variables and identifies a subset of those variables to statistically explain variance in the data set. Additionally, PCA can then be used to determine the contributions of specific variables to this variance. In the present study, PCA was used to compare groundwater sampling wells based on *dsrAB* gene diversity and groundwater geochemistry. Groundwater profiles were first analyzed to reveal geochemical variance between sampling wells, and then to identify the statistical contributions of specific chemical analytes (pH, NO_3^-, SO_4^{2-}, U, DO, Tc^{99}, Ni, TOC, NPOC) to this variance structure. Output data were plotted in two dimensions with the first principal component (PC1) on the *x*-axis and the second principal component (PC2) on the *y*-axis. The percentage variance explained by PC1 and PC2 is provided in parenthesis.

All *dsrAB* sequences described in this study have been deposited to the GenBank database under accession numbers AY885427–AY885558.

Results

Groundwater geochemistry

A summary of groundwater chemistry is presented in Table 1. PCA was used to first evaluate groundwater geochemical variability across sampling wells, and subsequently to rank specific analytes by their contribution to this variability (Fig. 1). FW-015, FW-010, FW-003 and FW-005 each had high component loading factors for PC1 (>0.73), whereas TPB-16 and FW-300 each had lower loading factors, 0.31 and 0.42, respectively. PC1 accounted for 45% of the total geochemical variance measured across sampling wells. Figure 1(a) portrays clear differences in groundwater geochemistry between sampling wells in the aquifer. TPB-16 and FW-300 were strongly weighted in PC2, 0.81 and 0.72, respectively.

PCA identified nickel, nonpurgable organic carbon (NPOC), and nitrate as the primary contributors (PC1 loading factor >0.9) of well-to-well geochemical variability (Fig. 1b). Total organic carbon (TOC) and Tc^{99} were also significant contributors, 0.78 and 0.7 respectively, whereas U only contributed weakly (0.2). All other geochemical variables received negative loading factors for PC1. A total of 54% of the total geochemical variance between sampling wells could be explained by these variables alone. All geochemical analytes contributed weakly to PC2.

dsrAB clone library characterization

dsrAB gene products of the expected size were successfully amplified from genomic DNA extracted from all FRC groundwater samples. PCR products were pooled from five independent amplification reactions per sampling well, cloned, and a total of 1878 colonies were recovered for detailed genetic analysis (Table 1). *dsrAB* gene diversity was evaluated at two levels; first, RFLP was used to resolve the entire *dsrAB* clone library. Second, comparative sequence analysis was performed for representative clones of each RFLP banding pattern observed.

RFLP analysis revealed that roughly 50% of all *dsrAB* clones recovered from each sampling well produced a unique RFLP pattern (Table 1). Interestingly, the average number of unique RFLP patterns recovered did not differ substantially among clone libraries from high-contaminant burden sites within the immediate perimeter of the S3 source ponds (FW-005, FW-010 and FW-015; mean $\pm$ SD $= 55 \pm 7$ patterns) or moderately burdened sites (FW-003, TPB-16; mean $\pm$ SD $= 56 \pm 10$) 275 m away from the S3 source ponds. Only slightly fewer (46%) clones from background site FW-300 yielded a unique RFLP gel banding pattern.

The similarity among *dsrAB* clone libraries is provided in Table 2. For all possible site pairings, over 59% of *dsrAB* clones were only sampled at a single well location. Only 39% of the *dsrAB* clones recovered from high-contaminant burden sites were sampled from multiple wells. On average, 144 common clones (SD $\pm$ 19; 129–166 common clones) were recovered from both high-contaminant burden sites and the moderate-contaminant burden well FW-003, whereas on average 188 common clones (SD $\pm$ 20; 167–207 common clones) were sampled from both high-contaminant burden sites and the background well FW-300. A total of 268 common clones were recovered from both the moderately burdened site FW-003 and the background well. TPB-16 was distinct; 99% of this *dsrAB* clone library was unique to this site.

Cluster analysis of all RFLP patterns revealed 136 distinct clone groupings consisting of identical or highly similar RFLP patterns. A representative clone was randomly selected from each RFLP group for subsequent analysis. By

Table 1. Summary of groundwater chemistry for Field Research Center groundwater sampling wells.

	Chemical analyte (mg L^{-1})									*dsrAB* clone libraries	
	pH	NO_3^-	SO_4^{2-}	U(pCi/L)	DO	Tc^{99}	Ni	TOC	NPOC	*n*	Unique clones (%)
High contaminant burden											
FW-010	3.52	44248.0	2.55	0.166	1.64	7190.0	17.5	173.0	73.5	355	205 (58)
FW-005	3.88	390.0	757.0	6.36	3.85	524.0	4.95	73.4	18.6	212	99 (47)
FW-015	3.37	10744.0	8.0	7.7	0.0	18500.0	8.8	64.9	33.3	317	187 (59)
Moderate contaminant burden											
FW-003	6.18	1608.0	15.2	0.005	2.31	135.0	0.013	102.0	13.1	338	165 (49)
TPB-16	6.31	5.5	74.2	1.13	0.75	36.0	0.003	62.6	2.24	334	211 (63)
Background											
FW-300	6.13	1.2	6.2	0.001	5.53	36.0	0.003	26.0	2.2	322	148 (46)

Groundwater sampling wells were categorized by contaminate burden, which was defined by relative contaminant concentrations and proximity to the source zone. Abbreviated designations were used for total uranium (U), dissolved oxygen (DO), total organic carbon (TOC), and nonpurgable organic carbon (NPOC). The total number of *dsrAB* clones recovered from each sampling well = *n*. The number of unique clones was determined restriction fragment length polymorphism (RFLP) analysis and the percentage of each clone library yielding a unique RFLP banding pattern is provided in parentheses.

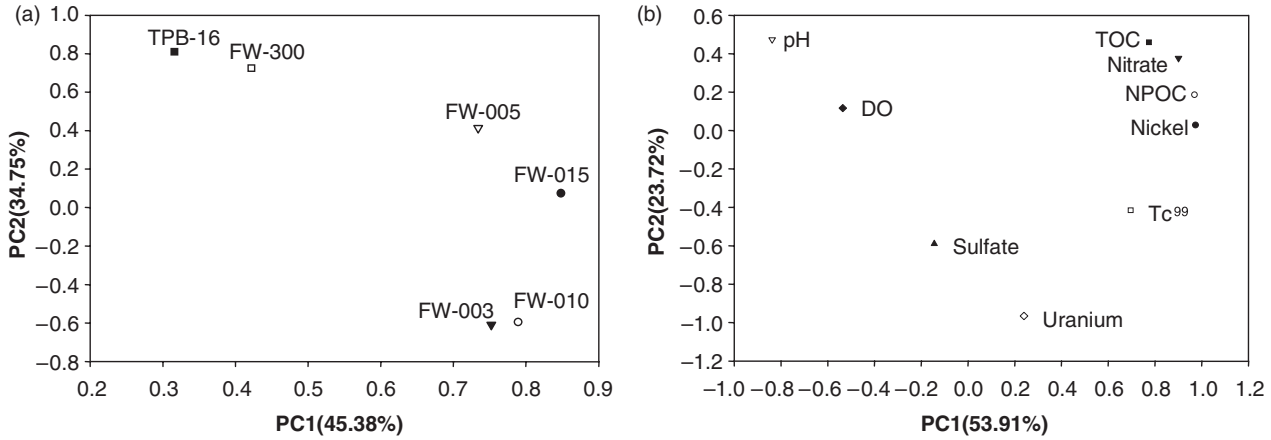

Fig. 1. Two-dimensional plots of principal components analysis describing the total measured geochemical variance across Field Research Center groundwater sampling wells (a) and the contribution of each measured geochemical analyte to the total measured chemical variability (b). Percent geochemical variability ascribed to PC1 (*x*-axis) and PC2 (*y*-axis) are provided in parenthesis for each axis.

evaluating the frequency of recovery of RFLP general group patterns across groundwater sampling wells, similarities in *dsrAB* community structure became more evident. Nine (FW-003-032, FW-003-269, FW-005-008, FW-005-298, FW-010-045, FW-010-274, FW-015-046, TPB-16-142, TPB-16-143) RFLP pattern types (or groups) were recorded for all six sampling wells. Clones FW-010-045 (clade 1) and TPB-16-143 (clade 7) represented the predominant RFLP pattern types within the entire *dsrAB* library, accounting for 11% and 6% of all *dsrAB* sequences recovered, respectively.

PCA of the major RFLP pattern types (136 groups) was used to discern sampling wells based on *dsrAB* gene composition (Fig. 2). Sampling wells FW-005 (high-contaminant burden site), FW-003 (moderate-contaminant burden site) and FW-300 (background) clustered tightly (PC1 $>$ 0.82), and wells FW-015 and FW-010 (both high-contaminant burden sites) clustered peripherally to this core grouping of sampling wells (PC1 $>$ 0.58). TPB-16 received a negative loading factor for PC1 and was strongly weighted by PC2, resulting in clear separation from all other sites.

Table 2. Percent similarity in *dsrAB* community structure between groundwater sampling wells at the Field Research Center. *dsrAB* nucleotide comparisons were based on restriction fragment length polymorphism banding pattern analysis

	High contaminant burden			Moderate contaminant burden		Background
	FW-005	FW-010	FW-015	FW-003	TPB-16	FW-300
FW-005		23	19	25	1	36
FW-010			18	24	0.7	31
FW-015				20	0	26
FW-003					0	41
TPB-16						0

Diversity of *dsrAB* genes

Nucleotide sequence was determined for 136 *dsrAB* clones, providing complete representation of all the unique RFLP patterns recovered. Phylogenetic reconstructions based on *dsrA* (data not shown), DsrA (data not shown), and *dsrAB* were highly conserved. The majority of all FRC sequences consistently fell into 10 clades composed exclusively of sequences from all six sampling wells that have little sequence similarity to known SRB (Fig. 3). Only clades 6 and 10 did not include *dsrAB* genes from TPB-16; all other clades included *dsrAB* sequences from all six groundwater sampling wells. A single *dsrAB* sequence was selected randomly from each FRC sequence clade for presentation and this designated sequence will be referenced for discussion of each sequence clade throughout the remainder of this paper.

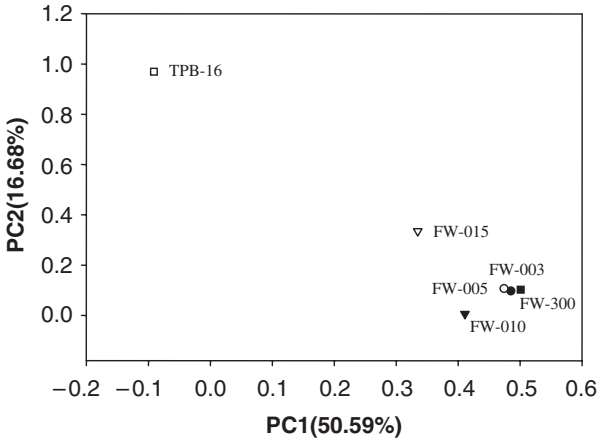

Fig. 2. Two-dimensional principal components plot describing *dsrAB* compositional variability across all Field Research Center groundwater sampling wells. Percent compositional variability ascribed to PC1 (*x*-axis) and PC2 (*y*-axis) are provided in parenthesis for each axis.

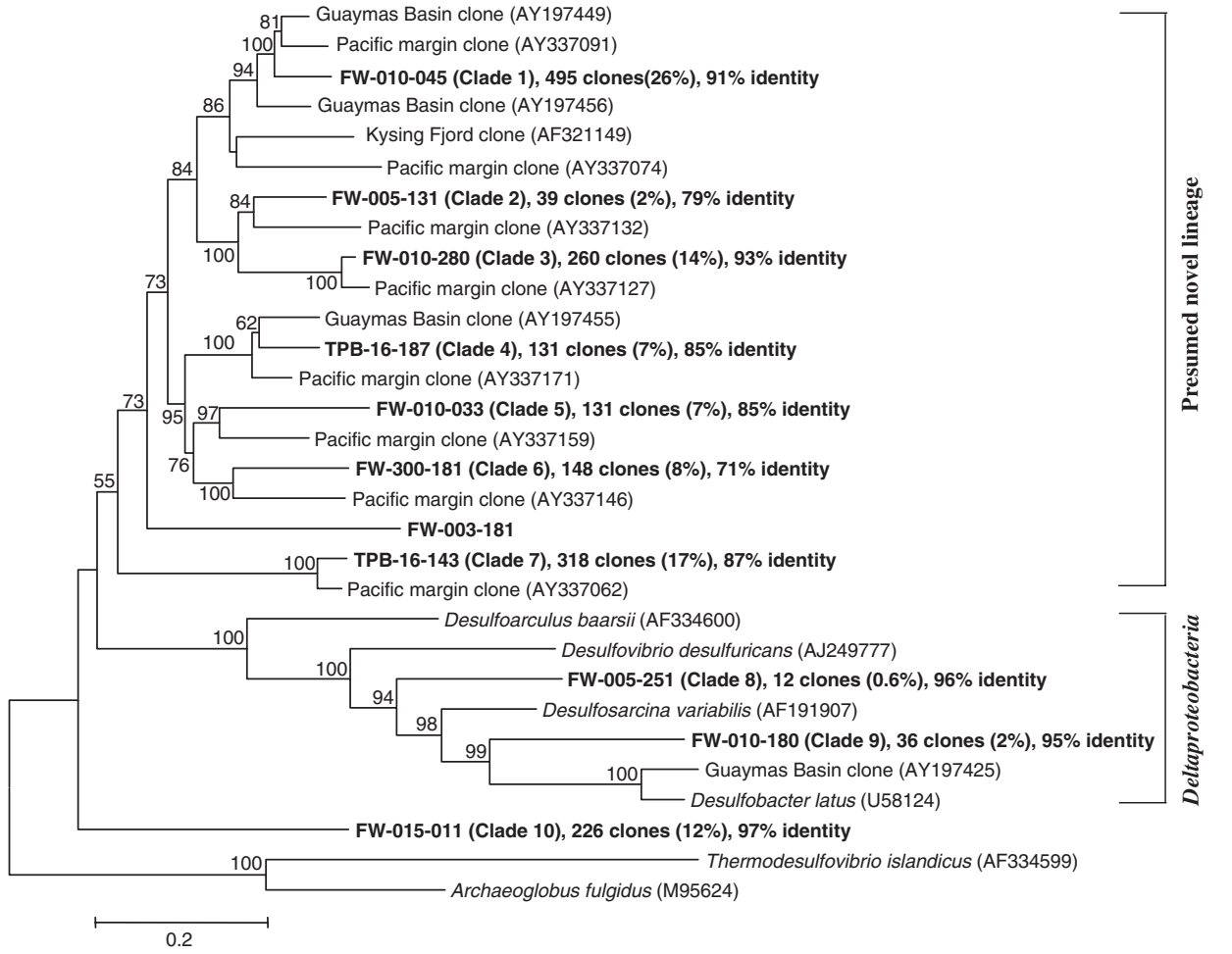

Fig. 3. Comparative sequence analysis of Field Research Center (FRC) groundwater *dsrAB* sequences and selected reference sequences from other different environmental systems and formally described sulfate reducing taxa. Sequence clades consisting only of FRC cloned *dsrAB* sequences were collapsed, and a single representative cloned sequence from each clade was selected for presentation. FRC groundwater cloned sequences are labeled by groundwater sampling well and arbitrary clone number. The total number of FRC *dsrAB* clones belonging to each collapsed sequence clade, percentage of the total *dsrAB* clone library (in parenthesis), and percent sequence homology within the clade is provided along side each clade representative sequence label. Branch order robustness was determined by bootstrap analysis (500 samplings), support values > 50% are shown.

Comparative sequence analysis for the FRC sequence clade representatives and *dsrAB* gene sequences from recognized SRB and selected environmental clone sequences is illustrated in Fig. 3. Sequence clades 1–7 (81% of the total *dsrAB* clone library) diverged sharply from *dsrAB* sequences from authentic SRB and seemingly belong to a novel, deep-branching lineage of SRB presently containing only environmental cloned sequences. The two predominant *dsrAB* sequence types belonged to this proposed novel *dsrAB* lineage and are represented by FW-010-045 in clade 1 and TPB-16-143 in clade 7. The overall sequence identity of this presumed novel *dsrAB* lineage was 61%. The closest *dsrAB* sequence from a recognized SRB was from *Desulfoarculus baarsii* but homology scores were $<28\%$ across the entire lineage. Sequences within this presumed divergent lineage were, however, closely affiliated with environmental clones from several different marine sedimentary habitats, namely Guaymas Basin (Dhillon *et al.*, 2003), the Pacific continental margin (Liu *et al.*, 2003) and Kysing Fjord sediments supporting CH_4 oxidation (Thomsen *et al.*, 2001). FRC sequence clade representatives showed 73–95% sequence identities to cloned sequences from these different marine habitats. Phylogenetic reconstructions with the FRC sequences were consistently rooted by authentic *dsrAB* sequences from *Thermodesulfovibrio islandicus* and *Archaeoglobus fulgidus*, supporting the supposition that these divergent sequences are likely to be true *dsrAB* gene sequences from putatively novel sulfate-reducing taxa.

FRC sequence clades 8 and 9 clustered most closely to the sequences from *Deltaproteobacteria*, although sequence

identity scores were weak. FW-005-251 (clade 8) and FW-010-180 (clade 9) were 53% identical to each other. Both sequence clades consistently clustered (48–64% identity) with the *dsrAB* lineage containing *Desulfofaba gelida*, *Desulfobotulus sapovorans*, *Desulfosarcina variabilis* and *Desulfococcus multivorans* (63% identity across genera), but sequence similarity scores suggests that these FRC *dsrAB* clades could represent new genera within this lineage.

FW-015-011 (clade 10) appeared to cluster more closely to sequences from the *Deltaproteobacteria* (identity scores $<$33%), but this phylotype is clearly quite different from all existing subgroups within this division. MEGABLAST searches against the NCBI database indicated that the closest *dsrAB* sequence match to FW-015-011 was that from the thermophilic sulfate reducing bacterium *Desulfacinum infernum,* but pair-wise sequence comparisons only supported 75% nucleotide identity. Unambiguous placement of this lineage must await comparison with more closely related gene sequences.

A single FRC *dsrAB* sequence (FW-003-181) was phylogenetically disparate and did not show significant homology to any of the FRC sequence clades or previously published NCBI database sequences. MEGABLAST searches across the NCBI database revealed that a Guaymas Basin clone (accession number AY197444) was most similar (52% identity) to FW-003-181. The percentage identity to other FRC environmental clone sequences belonging to the novel *dsrAB* lineage presented here was between 43–54%.

Discussion

Restriction fragment length polymorphism is a powerful technique for the fine-scale discrimination of gene sequences; relatively few nucleotide polymorphisms at key restriction sites are sufficient to change a gel banding pattern, signifying new gene diversity. In the current study, RFLP detection of *dsrAB* clone diversity was especially sensitive due to the use of four-base restriction endonucleases and the presence of a variable intergenic region between *dsrA* and *dsrB.* So although RFLP analysis of *dsrAB* clone libraries described impressive gene diversity, cluster analysis consolidated nearly 85% of this diversity, identifying only 136 major RFLP pattern types out of 1878 clones. Furthermore, the same phylotype sequences were consistently described for all well locations, revealing no major shifts in *dsrAB* community structure, including TPB-16. Several FRC *dsrAB* clades may ultimately be resolved by comparison with more relevant database sequences, although, despite all sequences being scored as unique by RFLP, many of these clades consisted of highly homologous gene sequences that were distributed throughout the aquifer. Comparative analysis of FRC *dsrAB* gene sequences indicates that SRB community composition was consistent between contaminated and uncontaminated sites in the Bear Creek Valley aquifer.

Resilience in community composition implies that these *dsrAB* phylogenetic populations may be insensitive to or capable of coping with high contaminant concentrations in FRC groundwater. Gillan *et al.* (2005) described similar results for members of the *Desulfosarcina–Desulfococcus* group, whose distributions along a contaminant gradient in shallow marine sediments failed to correlate with HCl-extractable metals or PCBs. Because of findings like these, considerable discussion has been devoted to the appropriateness of using molecular diversity assessments as an accurate indicator of the effects of anthropogenic stress on an ecosystem (Chapman, 1999). One reason for this debate, as noted above, is attributed to the differences in sensitivity of different molecular methodologies. Another complicating factor is that microbial diversity can be restored to predisturbance levels at sites that have a long history of contamination (Sandaa *et al.*, 2001; Ellis *et al.*, 2003; Gillan *et al.*, 2005). In this study, the same *dsrAB* phylotypes were sampled from contaminated and uncontaminated sites in the aquifer and *dsrAB* gene diversity did not correlate with contaminant burden. The Bear Creek Valley aquifer has been impacted by legacy nuclear waste for nearly 30 years and the results presented here give little indication that groundwater chemistry currently influences SRB community structure.

Lateral gene transfer of *dsrAB* has been documented (Klein *et al.*, 2001) and could also contribute to the proliferation of some *dsrAB* genotypes in this aquifer. Genetic variability and distribution patterns for *dsrAB* sequence clades 1, 3, 8, and 10 are particularly interesting in this regard. These clades were the largest clone groupings ($>$50% total clone library) and consisted of highly homologous *dsrAB* sequences (91–97% clade identity) recovered throughout the contaminated aquifer. Although it is possible that these sequences are representatives of authentic bacterial populations that are physiologically insensitive to the differences in the chemical environment encountered between sampling wells, an explanation by lateral gene transfer cannot be discounted.

TPB-16, a moderately contaminated site, was chemically most similar to the background well FW-300, but RFLP analysis consistently indicated a clear distinction of *dsrAB* genotypes from this site relative to all others. The significance of this sequence variation is uncertain, although TPB-16 *dsrAB* gene sequences were phylogenetically similar to those from other contaminated sites and the background site. One important distinguishing feature of TPB-16, is the use of a zero-valent iron reactive barrier for passive groundwater remediation. Resulting changes in groundwater chemistry and microbiology have been documented (Gu *et al.*, 2002). Within one year of installation, groundwater contaminant concentrations were significantly lowered,

reducing conditions established, and circumneutral groundwater pH obtained. At the same time as these chemical changes, microbial cell numbers increased by three- to 10-fold in groundwater and soil. Most notable was the apparent numerical dominance of sulfate reducing and denitrifying bacteria populations. Other studies have described enhanced sulfate reduction activity (Gu *et al.*, 1999; Phillips *et al.*, 2000) and contaminant biodegradation activity (Gandhi *et al.*, 2002) in Fe^0 barriers. No measurements of metabolic byproducts or metal redox speciation were taken in this study.

FRC sequence clades 1–7, clade 10, and FW-003-181 presumably represent a deeply branching, highly divergent lineage that is strongly different from *dsrAB* gene sequences from authentic SRB. Homologous *dsrAB* phylotype sequences were recently reported from the Pacific continental margin (Liu *et al.*, 2003), Guaymas Basin (Dhillon *et al.*, 2003), a deep-sea cold CH_4 seep (only partial *dsrA* sequences published, comparisons not shown; Fukuba & Naganuma, 2000), and CH_4 oxidizing Kysing Fjord sediments (Thomsen *et al.*, 2001). Petroleum-like hydrocarbons were considered as predominant electron donors at the Guaymas Basin site, and CH_4 was a seemingly important electron source in the deep-sea seep sediments and localized depths within the fjord sediments. A variety of potential carbon/electron donors are routinely measured in FRC groundwater, including halogenated aliphatics (i.e. chlorinated ethenes), aromatics (i.e. benzene), ketones (i.e. acetone), as well as carboxylic acids (i.e. acetate), but their potential contribution to SO_4^{2-} or metal reduction is currently unknown. At present this deeply branching lineage contains no cultivated members.

Recent literature suggests that because sulfate reducers are consistently detected at metal-contaminated sites or environments where uranium is naturally abundant, these bacteria may contribute to metal geochemistry (Chang *et al.*, 2001; Nevin *et al.*, 2003; Suzuki *et al.*, 2003). In accordance with these studies, we have described an impressive catalogue of new *dsrAB* gene sequences for a heavily contaminated subsurface aquifer, but compared to the background site, metal contamination has had no discernable imprint on *dsrAB* gene diversity or distributions in this system. The nuclear legacy waste in this aquifer does not appear to impose strong negative selection against native SRB populations or positive selection favoring the proliferation of new SRB populations. Based on the data presented here, there is no clear indication that the SRB would necessarily be major catalysts for metal cycling in this system. Confirmation, however, will require examination of the functional attributes of these SRB populations, as the molecular methods deployed here are not quantitative and cannot discriminate between active, inactive or dead cells. Future investigations will explore the identity and physiology of these potentially unique sulfate reducers to determine whether these bacteria are capable of growth and metabolism in this heavily contaminated extreme environment. Comparative physiology between cognate populations isolated from contaminated and uncontaminated sites should allow for unambiguous determination of specific adaptations that may have been acquired to enable remarkable tolerance and persistence in metal-contaminated regions of this aquifer.

Acknowledgements

We gratefully acknowledge Gary King and anonymous reviewers whose input substantially improved this manuscript. This research was supported by The United States Department of Energy under the Natural and Accelerated Bioremediation Research Program of the Office of Biological and Environmental Research, Office of Science. Oak Ridge National Laboratory is managed by UT-Battelle, LLC for DOE under contract # DE-AC05-96OR22464. The authors CEB and XL contributed equally to this work.

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