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Detection and Quantification of *Geobacter lovleyi* Strain SZ: Implications for Bioremediation at Tetrachloroethene- and Uranium-Impacted Sites▽†

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Geobacter lovleyi* strain SZ reduces hexavalent uranium, U(VI), to U(IV) and is the first member of the metal-reducing *Geobacter* group capable of using tetrachloroethene (PCE) as a growth-supporting electron acceptor. Direct and nested PCR with specific 16S rRNA gene-targeted primer pairs distinguished strain SZ from other known chlorinated ethene-dechlorinating bacteria and closely related *Geobacter* isolates, including its closest cultured relative, *G. thiogenes*. Detection limits for direct and nested PCR were approximately 1×10^6 and 1×10^4 16S rRNA gene copies per μl of template DNA, respectively. A quantitative real-time PCR (qPCR) approach increased the sensitivity to as few as 30 16S rRNA gene copies per μl of template DNA but was less specific. Melting curve analysis and comparison of the shapes of amplification plots identified false-positive signals and distinguished strain SZ from *G. thiogenes* when analyzed separately. These indicators were less reliable when target (strain SZ) DNA and nontarget (*G. thiogenes*) DNA with high sequence similarity were mixed, indicating that the development of qPCR protocols should not only evaluate specificity but also explore the effects of nontarget DNA on the accuracy of quantification. Application of specific tools detected strain SZ-like amplicons in PCE-dechlorinating consortia, including the bioaugmentation consortium KB-1, and two chlorinated ethene-impacted groundwater samples. Strain SZ-like amplicons were also detected in 13 of 22 groundwater samples following biostimulation at the uranium- and chlorinated solvent-contaminated Integrated Field-Scale Subsurface Research Challenge (IFC) site in Oak Ridge, TN. The numbers of strain SZ-like cells increased from below detection to $2.3 \times 10^7 \pm 0.1 \times 10^7$ per liter groundwater, suggesting that strain SZ-like organisms contribute to contaminant transformation. The *G. lovleyi* strain SZ-specific tools will be useful for monitoring bioremediation efforts at uranium- and/or chlorinated solvent-impacted sites such as the Oak Ridge IFC site.

Chlorinated ethenes such as tetrachloroethene (PCE) and trichloroethene (TCE) are common groundwater pollutants due to their extensive use for industrial and military applications (17). Hexavalent uranium, U(VI), has been released into the environment as a consequence of weapons-grade uranium production and milling (20, 52). Uranium processing typically requires chlorinated solvents, such as PCE and TCE; therefore, U(VI) and chlorinated ethene contamination often coexist (e.g., at the Integrated Field-Scale Subsurface Research Challenge [IFC] site [formerly the Field Research Center] in Oak Ridge, TN [http://www.esd.ornl.gov/nabirfrc]) (48, 50). Due to negative human health impacts, both chlorinated ethenes and uranium are regulated by the U.S. Environmental Protection Agency (EPA). Considerable effort has been devoted to elucidate the fate of these contaminants and to identify microbial populations involved in their transformation and detoxification (19, 43, 49). Although a number of phylogenetically diverse PCE- or U(VI)-reducing bacteria have been obtained from various environments (21, 22, 43, 49), none of the isolates were reported to mediate both activities. Only the recently described isolate *Geobacter lovleyi* strain SZ combines the ability to reductively dechlorinate PCE and reduce soluble U(VI) to sparingly soluble U(IV) (40, 45). Interestingly, strain SZ reduces PCE and U(VI) concomitantly (45), making this organism a promising candidate for bioremediation at mixed-waste sites such as the Oak Ridge IFC site. *Geobacter* spp. are commonly found in freshwater sediments and subsurface environments, but the distribution of members of the recently proposed dechlorinating *Geobacter* clade (45) is unclear. The goals of this study were to design specific and quantitative 16S rRNA gene-based approaches for assessing the environmental

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TABLE 1. Primers used to detect and quantify *Geobacter lovleyi* strain SZ and alignment of 16S rRNA gene sequence fragments of strain SZ with corresponding regions of closely related species

Organism	Primer sequence alignment (5′ → 3′)[a]		
	Geo196F	Geo535R	Geo999R
G. lovleyi	GAATATGCTCCTGATTC	TAAATCCGAACAACGCTT	ACCCTCTACTTTCATAG
G. thiogenes	GAATATGCTCTTGATCT	TAAATCCGAACAACGCTT	ACCCCTCGCTTTCACGA
G. metallireducens	TTCGGGCCTTTTGTCAC	TAATTCCGAACAACGCTT	ACCCCCTACTTTCATAG
G. sulfurreducens	TTCGGGCCTCCTGTCTT	TAATTCCGAACAACGCTT	ACCCCTCCGTTTCGGGA
D. michiganensis	TCGGGTCCTACTGTCAT	TAATTCCGAACAACGCTT	ACCCCTATGTTTCCATA

[a] Underlining indicates mismatches. The primers correspond to the following positions on the *E. coli* 16S rRNA gene sequence (GenBank accession number U00096.2): Geo196F, bp 208 to 224; Geo535R, bp 563 to 546; and Geo999R, bp 1025 to 1009. The positions were determined using the MegAlign program of the Lasergene software package (DNA Star, Inc., Madison, WI).

distribution and abundance of strain SZ-like *Geobacter* spp. in uranium- and chlorinated ethene-contaminated sites and to explore the value of the new tools for bioremediation-monitoring efforts.

MATERIALS AND METHODS

Sources of DNA. DNA was isolated from pure cultures, PCE-dechlorinating enrichment cultures, bioaugmentation consortia, and groundwater and aquifer materials from contaminated sites. The pure cultures included *G. lovleyi* strain SZ (DSM 17278) (45), *G. thiogenes* (formerly *Trichlorobacter thiogenes*) (ATCC BAA-34, kindly provided by B. Griffin, Michigan State University) (8, 31), *G. sulfurreducens* (DSM 12127, kindly provided by K. Nevin, University of Massachusetts) (4), *G. metallireducens* (DSM 7210, kindly provided by K. Nevin) (26), *Desulfuromonas michiganensis* strain BB1 (DSM 15941) (47), *Anaeromyxobacter dehalogenans* strain 2CP-C (ATCC BAA-259) (39), *Sulfurospirillum* (formerly *Dehalospirillum*) *multivorans* (DSM 12446) (28, 41), *Dehalococcoides* sp. strain FL2 (14), *Dehalococcoides* sp. strain BAV1 (13), and *Desulfitobacterium* sp. strain Viet1 (23, 25). The *Geobacter* isolates were grown as described by Sung et al. (45). Strain BB1 and strain 2CP-C were grown as described by Sung et al. (47) and Sanford et al. (39), respectively, and all other pure cultures were grown as described by Amos et al. (1). R. Daprato (Georgia Institute of Technology) kindly provided genomic DNA and a plasmid containing a single copy of the 16S rRNA gene of *Dehalobacter restrictus* (DSM 9455) (15). Genomic DNA was obtained from three PCE-to-ethene dechlorinating enrichment cultures derived from chlorinated ethene-contaminated aquifer materials (the Hydrite site, Cottage Grove, WI; the TRW site, Minerva, OH; and the FMC site, San Jose, CA) (44); one PCE-to-*cis*-1,2-dichloroethene (*cis*-DCE) dechlorinating enrichment culture derived from TCE-contaminated aquifer materials collected at the East Gate Disposal Yard in Ft. Lewis, WA (34); and one PCE-to-ethene dechlorinating mixed culture derived from Kalamazoo River (MI) sediment (44). BioDechlor INOCULUM, a PCE-to-ethene dechlorinating bioaugmentation inoculum (37), was grown as described previously (1) to obtain genomic DNA. Genomic DNA of the bioaugmentation inoculum KB-1 (10, 11, 30) was kindly provided by M. Duhamel, University of Toronto. DNA was also obtained from groundwater collected within a TCE plume originating from an industrial site near Milledgeville, GA, and from aquifer material collected at the East Gate Disposal Yard in Ft. Lewis, WA (34). Twenty-two groundwater samples were obtained from the uranium-contaminated Oak Ridge IFC site. Samples were collected from wells FW016 and FW029 of area 1 (16) and well TPB16 of area 2. Area 3 groundwater samples were from the unconditioned and nonstimulated control well FW106 (27 May 2004) (51) and from a successful pilot-scale uranium bioreduction demonstration plot (50, 51). The pilot-scale demonstration used a nested-cell approach comprising a biostimulated inner recirculation loop that periodically received an electron donor (i.e., ethanol) and an outer recirculation loop that minimized interactions between the low-pH and high-nitrate aquifer groundwater and the chemically and biologically conditioned groundwater in the biostimulated, inner loop (50, 51). The four-well recirculation system consisted of an outer loop injection well (FW024), an outer loop extraction well (FW103), an inner loop injection well (FW104), and an inner loop extraction well (FW026). The system also contained three multilevel sampling wells: FW100, FW101, and FW102. Specific multilevel sampling well depths are designated as outlined by Wu et al. (50). Periodic addition of ethanol to the inner recirculation loop began on 7 January 2004 (51). Groundwater samples were obtained on four occasions during the first 6 months of periodic biostimulation with ethanol: 2 February 2004 (wells FW103, FW100-2, FW100-3, FW101-3, and FW104); 5 February 2004 (well FW024); 27 May 2004 (wells FW026 and FW101-2); and 16 June 2004 (well FW102-3). Additional samples were obtained 1 to 1.5 years after the onset of periodic ethanol biostimulation as follows: 25 May 2005 (well FW104); 4 August 2005 (wells FW104 and FW102-2); and 5 August 2005 (wells FW026, FW101-1, FW102-1, FW102-4, FW100-1, and FW100-4). Eleven of the 22 samples came from wells FW029, FW104, FW026, FW101-3, FW101-1, FW101-2, FW102-2, and FW102-3, located in subsurface regions affected by biostimulation (i.e., ethanol addition), while the remaining samples came from wells FW016, TPB16, FW106, FW024, FW103, FW100-2, FW100-3, FW102-1, FW102-4, FW100-1, and FW100-4, located in regions not influenced or only marginally affected by biostimulation (16, 50, 51).

DNA isolation. Genomic DNA was extracted from 5 to 10 ml of chlorinated ethene-dechlorinating pure and mixed cultures, as well as from cultures of *G. thiogenes*, *G. metallireducens*, and *G. sulfurreducens*, by use of a QIAamp DNA mini kit (QIAGEN, Valencia, CA) with modifications as described previously (1, 36). For quantitative real-time PCR (qPCR) standards, a 16S rRNA gene of strain SZ was cloned into plasmid Topo-TA pCR2.1 (Invitrogen, Carlsbad, CA) as described previously (18); plasmids containing strain SZ's 16S rRNA gene (pSZ16S) were extracted from *Escherichia coli* host cells by use of a QIAprep spin miniprep kit (QIAGEN) (36). Community genomic DNA was isolated from 1.8 to 1,715 liters of Oak Ridge IFC site groundwater and collected in final volumes of 10 to 450 μl as described previously (53); for qPCR, the DNA was diluted 1:10 or 1:20 before analysis. Genomic DNA was obtained from 1 liter of Milledgeville site groundwater and from 40 ml of a TCE-fed Ft. Lewis enrichment culture by use of a MoBio ultra clean water DNA kit (Jefferson City, MO) (36). DNA from 10 g of Ft. Lewis soil was extracted using a PowerMax soil DNA isolation kit (MoBio). Purified DNA was stored at −20°C until analysis.

PCR primers and conditions. Specific PCR primer pairs were designed using Oligo design and analysis tools (Integrated DNA Technologies, Coralville, IA; http://www.idtdna.com/SciTools/SciTools.aspx?cat=DesignAnalyze) based on the nearly complete 16S rRNA gene sequence of strain SZ (GenBank accession number AY914177). The 16S rRNA gene sequences of strain SZ and related species, including *G. thiogenes* (GenBank accession number AF223382), *G. sulfurreducens* (GenBank accession number U13928), *G. metallireducens* (GenBank accession number L07834), and *Desulfuromonas michiganensis* strain BB1 (GenBank accession number AF357915), were aligned using the MegAlign program of the Lasergene software package (DNA Star, Inc., Madison, WI). The sequences of the selected primers, Geo196F and Geo999R, and mismatches between the primer sequences and the sequences of 16S rRNA gene fragments from closely related organisms are shown in Table 1. The *Geobacteraceae*-targeted primer Geo564F described by Cummings et al. (5) was modified to produce primer Geo535R (Table 1). The expected sizes of the Geo196F/Geo999R and Geo196F/Geo535R amplicons are 820 and 357 bp, respectively.

To experimentally verify primer specificity, purified genomic DNA of close *Geobacter* relatives and selected reductively dechlorinating species (*D. michiganensis* strain BB1, *Anaeromyxobacter dehalogenans* strain 2CP-C, *Sulfurospirillum multivorans*, *Dehalobacter restrictus*, *Dehalococcoides* sp. strain FL2, *Dehalococcoides* sp. strain BAV1, and *Desulfitobacterium* sp. strain Viet1) was subjected to PCR with both Geo196F/Geo999R and Geo196F/Geo535R primer pairs. The reaction mixture contained 2 μl of 10× PCR buffer (Applied Biosystems [ABI], Foster City, CA), 2 mM $MgCl_2$, 0.13 mg of bovine serum albumin/ml (Promega, Madison, WI), 200 μM of each deoxynucleoside triphosphate (ABI), 2.5 U of AmpliTaq polymerase (Gibco BRL, Gaithersburg, MD), 100 nM of each primer, and 1 μl of template DNA (10 to 20 ng/μl) in a total reaction volume of

20 μl. Amplification with a gradient thermocycler (Eppendorf Mastercycler gradient 5331; Eppendorf, Hamburg, Germany) determined optimum annealing temperatures of 50 and 53°C for primer pairs Geo196F/Geo535R and Geo196F/Geo999R, respectively. The following temperature program was used for the designed primer pairs: 94°C for 2.2 min, 30 cycles of 94°C for 30 s, 50°C or 53°C for 45 s, and 72°C for 2.2 min, followed by 6 min at 72°C. For nested PCR, an initial amplification was performed with bacterial primer pair 8F and 1514R followed by a second round of PCR with the strain SZ-specific primer pairs and using 1:50 dilutions of the amplicons obtained in the first round of PCR as template DNA (24). PCR products were visualized in 1% agarose gels in Tris-acetate-EDTA buffer stained in an aqueous ethidium bromide solution (1 μg/ml). Select amplicons obtained with the Geo196F/Geo999R primer pair were purified (QIAquick PCR purification kit; QIAGEN) and partially sequenced using an ABI 3100 genetic analyzer (ABI).

The detection limits (i.e., sensitivities) of direct and nested PCR with the Geo196F/Geo999R primer pair were determined by performing PCR on a 10-fold dilution series of *G. lovleyi* strain SZ genomic DNA. The gene targets in the highest dilution were quantified via qPCR analysis, and the DNA concentrations in subsequent dilutions were calculated based on this estimate. The reported detection limits are the lowest dilutions that yielded a visible band in ethidium bromide-stained gels.

qPCR analysis. qPCR analysis to quantify *G. lovleyi* strain SZ was performed using SYBR green-based detection chemistry and the Geo196F/Geo535R primer pair. The reaction mixture contained 15 μl of Power SYBR green PCR master mix (ABI), 300 nM of each primer, and 3 μl of template DNA in a total reaction volume of 30 μl. The PCR temperature program was as follows: 2 min at 50°C, 15 min at 95°C followed by 40 cycles of 30 s at 94°C, 30 s at 50°C, and 30 s at 72°C. qPCR was carried out with an ABI 7500 fast real-time PCR system, and melting curve analysis was performed with the default settings of the ABI software from 67°C to 95°C. Standard curves were generated following a procedure outlined by Ritalahti et al. (36), which used a 10-fold dilution series of quantified plasmid (concentrations determined spectrophotometrically at 260 nm) carrying a single copy of strain SZ's 16S rRNA gene (pSZ16S). To identify false positives, template DNA was replaced with sterile water (i.e., no-template controls). The genome of strain SZ contains two 16S rRNA gene copies (DOE Joint Genome Institute); therefore, dividing gene copy numbers by a factor of 2 yielded the cell numbers. The specificity of the Geo196F/Geo535R primer pair in the qPCR approach was evaluated with triplicate samples of purified genomic DNA of *G. lovleyi* strain SZ (12.5 ng/μl), *G. thiogenes* (16.1 ng/μl, 1.61 ng/μl, and 0.16 ng/μl), *G. sulfurreducens* (1.9 ng/μl), and *G. metallireducens* (6.9 ng/μl). To determine the influence of DNA from closely related species on the quantification of strain SZ, qPCR analysis was performed on DNA mixtures (see the supplemental material) with increasing amounts of *G. thiogenes* DNA. To determine if sample dilution affected strain SZ quantification, qPCR was performed on undiluted and 1:10-diluted strain SZ DNA.

RESULTS AND DISCUSSION

Specific, sensitive, and quantitative detection of strain SZ. With strain SZ DNA as the template, the designed primer pairs Geo196F/Geo999R and Geo196F/Geo535R yielded amplicons of the expected sizes (820 bp and 357 bp, respectively) and sequences. In direct and nested PCR, both primer pairs distinguished strain SZ from related species, including *G. thiogenes*, *G. sulfurreducens*, *G. metallireducens*, and *D. michiganensis* strain BB1, and the other reductively dechlorinating bacteria tested. Direct PCR with the Geo196F/Geo999R primer pair required $>10^6$ 16S rRNA gene copies per μl of template DNA to produce a visible band in ethidium bromide-stained agarose gels, whereas the nested-PCR approach was 2 orders of magnitude more sensitive. Although the primer pair Geo196F/Geo999R did not achieve the sensitivity reported for other 16S rRNA gene-targeted primers (e.g., see reference 24), the strain SZ-specific PCR approach proved valuable in evaluating the distribution of strain SZ-like organisms in environmental samples (see below). The qPCR approach was significantly more sensitive and had a quantification limit of ~30 16S rRNA gene copies per μl of template DNA. The qPCR approach produced linear standard curves ranging from 8.4×10^1 to 8.4×10^8 16S rRNA gene copies per PCR ($R^2 = 0.9991$; amplification efficiency of 1.80 [35]). The qPCR quantification limit reported herein is consistent with the quantification limits reported for other qPCR target genes (e.g., *Dehalococcoides* 16S rRNA and reductive dehalogenase genes [36]) and slightly lower than the quantification limit observed with another *Geobacter*-targeted qPCR protocol (10). The latter method used primer pair Geo73f/Geo485r to target a *Geobacter* organism whose 16S rRNA gene sequence (GenBank accession number AY780563) shares 99% identity with that of *G. lovleyi* strain SZ (10). This primer pair does not contain the discriminating mismatches presented in Table 1 and hence would not distinguish between *G. lovleyi* and *G. thiogenes*.

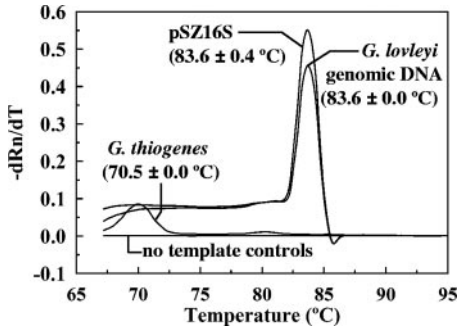

FIG. 1. Melting curve analysis following qPCR for samples of *G. lovleyi* strain SZ (genomic DNA or plasmid DNA with a 16S rRNA gene insert of strain SZ [pSZ16S]), samples of *G. thiogenes* (genomic DNA), and no-template controls. The melting curves represent averages of triplicate qPCR reactions for each sample, and average T_m (±standard deviations) for each sample are listed in parentheses. −dRn/dT represents the negative derivate of the reported fluorescent signal (Rn) with respect to temperature (T). The T_m for the target amplicon is at the maximum rate of change (i.e., the greatest −dRn/dT) for each melting curve.

In qPCR analysis, the shapes of the amplification (i.e., fluorescent signal intensity) plots (not shown) and the melting curves (Fig. 1) were similar for triplicate samples of genomic DNA from strain SZ compared to the plasmid (pSZ16S) standards. Melting (i.e., disassociation) temperatures of PCR amplicons depend mainly on amplicon size and GC content and can be used as an additional diagnostic tool to verify target sequence amplification (2, 3, 38). As shown in Fig. 1, the average melting temperatures (T_m) for the amplicons produced with genomic and plasmid (pSZ16S) DNA of strain SZ were 83.6 ± 0.0°C and 83.6 ± 0.4°C, respectively. These data agree well with the estimated T_m of 85°C calculated with an oligonucleotide properties calculator (version 3.13; http://www.basic.northwestern.edu/biotools/oligocalc.html) for the 357-bp amplicon. Minimal amplification (i.e., below the quantification limit) was observed with triplicate samples of *G. sulfurreducens* and *G. metallireducens* genomic DNA, and no amplification was observed without template DNA. Some amplification (i.e., fluorescence) was observed with *G. thiogenes*, although the shape of the amplification plot was markedly different than the shape of the plots for strain SZ. In fact, the software available with the ABI 7500 fast real-time PCR system failed to quantify samples of *G. thiogenes* due to a noisy baseline. The melting curves for samples of *G. thiogenes*, with an average T_m of

70.5 ± 0.0°C, were markedly different than the curves for strain SZ (Fig. 1), and the experimental T_m (70.5 ± 0.0°C) did not agree with the T_m of 84°C predicted for *G. thiogenes*. Similar melting curves and temperatures were obtained when 1:10 and 1:100 dilutions of the genomic DNA of *G. thiogenes* were used as templates in qPCR. When qPCR products were analyzed in agarose gels, amplicons of the expected size (357 bp) were observed for strain SZ, but no amplicons were produced with *G. thiogenes* template DNA. Fluorescence was not observed with strain SZ or *G. thiogenes* DNA when either the forward (Geo195F) or the reverse (Geo535R) primer was omitted from the PCR. Although some amplification of *G. thiogenes* DNA occurred with the described qPCR approach, multiple lines of evidence (e.g., melting curves, amplification plots, and noisy baselines) distinguished *G. lovleyi* strain SZ from *G. thiogenes* during qPCR analysis. Similar discrimination of closely related organisms by melting curve analysis has previously been described for distinguishing and identifying medically relevant microorganisms (summarized by Robinson et al. [38]). Hence, melting curve analysis may also be a useful tool to differentiate environmentally relevant bacteria with highly similar target sequences, potentially allowing for high-resolution discrimination (3).

Although the qPCR approach clearly identified false-positive signals, *G. thiogenes* DNA influenced the quantification of strain SZ when DNA from both isolates was present in the same sample. In the absence of *G. thiogenes* DNA, the average estimate for the number of strain SZ cells per ml of a PCE-grown culture was $1.50 \times 10^8 \pm 0.11 \times 10^8$ (see the supplemental material). The estimated cell numbers increased linearly ($R^2 = 0.971$) in the presence of increasing concentrations of *G. thiogenes* DNA by up to 1.78-fold (see the supplemental material). A 10-fold dilution of pure strain SZ template DNA did not have a significant effect on the cell number estimate, indicating that the observed effect of *G. thiogenes* DNA was not due to dilution of strain SZ DNA. The increased estimate for strain SZ in the presence of *G. thiogenes* is likely related to the nonspecific fluorescent signal produced from the *G. thiogenes* DNA. The melting curves following qPCR with mixtures of strain SZ and *G. thiogenes* DNA did not contain the characteristic peak for *G. thiogenes* (~70.5°C) (Fig. 1) but only the characteristic peak for strain SZ (average T_m of 83.9 ± 0.3°C). These results suggest that qPCR overestimates the abundance of strain SZ in the presence of *G. thiogenes* DNA and that melting curve analysis cannot identify when the overestimation occurs (i.e., when *G. thiogenes* and strain SZ DNA occur concomitantly in a sample). Hence, the development of qPCR protocols and other quantitative, nucleic acid-based tools should not only evaluate the specificity of the approach but also explore the effects of nontarget DNA, including DNA with high sequence similarity to the target DNA sequence, on the accuracy of quantification (27).

Application of the strain SZ-specific detection tools. Direct PCR with the Geo196F/Geo999R primer pair detected strain SZ-like organisms in the PCE-to-ethene dechlorinating Hydrite enrichment culture (not shown). With acetate provided as the electron donor, the Hydrite culture accumulated *cis*-DCE, whereas complete reductive dechlorination to ethene occurred in cultures amended with hydrogen (44). This dechlorination pattern is consistent with dechlorinator physiology: strain SZ couples either acetate or hydrogen oxidation to the reduction of PCE and TCE to *cis*-DCE (45), and *Dehalococcoides* sp. strain GT, which was isolated from the Hydrite culture (46), requires hydrogen as the electron donor to drive the process to completion (i.e., ethene formation). No amplification with the Geo196F/Geo999R primer pair was observed with DNA samples from three other PCE-to-ethene dechlorinating mixed cultures in either direct or nested PCR (not shown). Direct PCR produced strain SZ-like amplicons with DNA from the KB-1 bioaugmentation consortium, but no amplicons were obtained with DNA from Bio-Dechlor INOCULUM, even with nested PCR. Recent studies have demonstrated growth of the SZ-like organism in the KB-1 consortium during PCE and TCE dechlorination to *cis*-DCE (9–11). Strain SZ-like organisms were detected in both groundwater samples from a TCE-impacted aquifer near Milledgeville, GA. Dechlorination of TCE to stoichiometric amounts of *cis*-DCE was observed in microcosms and occurred in situ after biostimulation (42), suggesting that strain SZ-like organisms were stimulated and played a role in the observed dechlorination. No SZ-specific signals were obtained with DNA extracted from TCE-contaminated Ft. Lewis soil. However, PCR amplification with the Geo196F/Geo999R primer pair occurred with DNA obtained from a TCE- and lactate-amended enrichment culture derived from the Ft. Lewis soil, and qPCR estimated $4.34 \times 10^6 \pm 0.96 \times 10^6$ strain SZ-like cells per ml of culture. Additionally, three 16S rRNA gene sequences with 96 to 99% similarity to that of strain SZ were detected in a clone library constructed with DNA extracted from the Ft. Lewis enrichment culture (34).

Detection of strain SZ-like organisms in chlorinated ethene-dechlorinating enrichment cultures, in the KB-1 bioaugmentation consortium, and in environmental samples from chlorinated ethene-impacted aquifers, coupled with the fact that previous studies detected SZ-like 16S rRNA gene sequences in PCE-to-ethene dechlorinating mixed cultures and contaminated aquifers, suggests that dechlorinating *Geobacter* spp. may be relevant contributors to chlorinated ethene detoxification (6, 7, 9–11, 29). Apparently, chlorinated ethene-dechlorinating *Geobacter* species are not rare in the environment, thus expanding our understanding of the physiological versatility of the *Geobacteraceae*. Additional dechlorinating *Geobacter* isolates are needed to refine the parameters delineating the putative dechlorinating clade within the *Geobacteraceae* (45).

Detection of strain SZ at the Oak Ridge IFC site. Strain SZ-like organisms were detected in community genomic DNA extracted from 13 of 22 Oak Ridge IFC site groundwater samples. Specific amplicons were obtained with direct PCR (not shown) and nested PCR (Fig. 2A) from samples of wells FW026, FW101-2, and FW102-3, located within the area 3 biostimulated zone (50, 51). Nested PCR yielded an additional positive signal with DNA collected from well FW029 (area 1), which was also influenced by biostimulation (Fig. 2A) (16). Sequence analysis of at least 350 bp of all amplicons confirmed sequence identity with the 16S rRNA gene sequence of strain SZ. No amplification occurred (Fig. 2A) with samples from wells FW016 (area 1), TPB16 (area 2), and FW106 (area 3), which are located in areas that received no biostimulation treatment.

As depicted in Fig. 2B, strain SZ-like organisms could also be enumerated in various groundwater samples collected

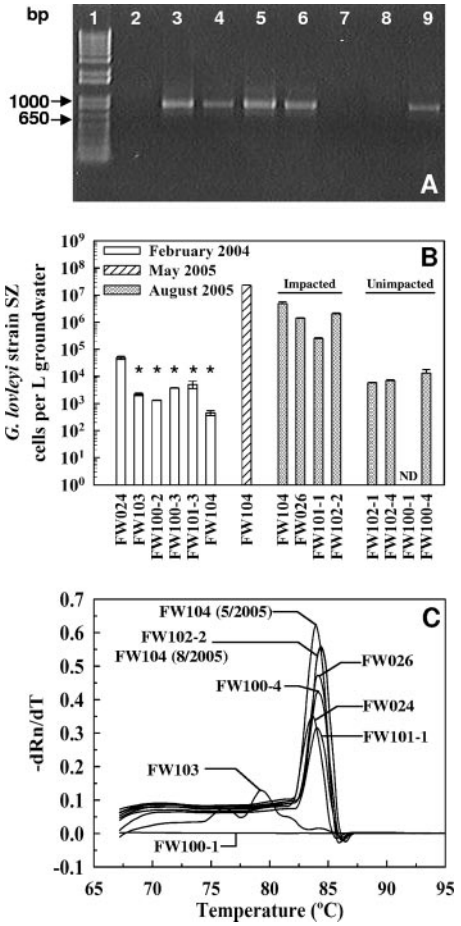

FIG. 2. (A) Detection of strain SZ-like organisms via nested PCR in chlorinated ethene- and uranium-contaminated Oak Ridge IFC site groundwater taken from regions impacted by biostimulation (wells FW026 [lane 3], FW029 [lane 4], FW101-2 [lane 5], and FW102-3 [lane 6]) or not impacted by biostimulation (wells FW016 [lane 2], FW106 [lane 7], and TPB16 [lane 8]). Lane 1 is the DNA size marker (Invitrogen), and lane 9 is genomic DNA of strain SZ. (B) Average *G. lovleyi* strain SZ cell numbers per liter of Oak Ridge IFC site groundwater in a pilot-scale uranium bioreduction demonstration plot. Samples were taken approximately 1 month after the initial ethanol amendment (February 2004) and in May and August 2005, after 1 to 1.5 years of periodic ethanol biostimulation. The samples from August 2005 came from aquifer regions impacted or not impacted by biostimulation, as indicated. Error bars indicate standard deviations of triplicate qPCR reactions and are not shown when they are too small to depict. The asterisks indicate that the cell number estimates were extrapolated values outside the range of accurate quantification and that the melting curves did not contain the characteristic peak for strain SZ (see text for details). ND, fluorescence was not detected. (C) Melting curves following qPCR for representative samples from the Oak Ridge IFC site. The melting curves represent averages of triplicate qPCR reactions for each sample. For an explanation of −dRn/dT, see the legend for Fig. 1.

within the uranium bioreduction plot at the Oak Ridge IFC site (50, 51). In February 2004, approximately 1 month after the initial amendment of ethanol to the inner recirculation loop, the number of strain SZ cells was less than 5×10^4 per liter groundwater (Fig. 2B). Minimal amplification (i.e., fluorescence) was observed in most of the samples from February 2004 (Fig. 2B), and cell number estimates for these samples were extrapolated from the linear qPCR standard curve. These estimates must be interpreted cautiously since the fluorescent signals were below the limit for accurate quantification (~30 16S rRNA gene copies per μl of template DNA; see above). The melting curves for these samples did not have the characteristic peak for strain SZ, but instead several smaller peaks were visible (Fig. 2C). All samples that could be quantified accurately had melting curves with the characteristic peak for strain SZ (Fig. 2C). These results demonstrate that melting curve analysis was useful to distinguish nonspecific amplification and identify false-positive signals with environmental samples. Samples taken following biostimulation indicated substantial growth of strain SZ-like organisms (Fig. 2B). For example, cell numbers in samples from the inner recirculation loop injection well (FW104) increased by over 3 orders of magnitude from February 2004 to May and August 2005 (Fig. 2B). The samples from May and August 2005 were taken 1 to 1.5 years after the onset of periodic ethanol addition to the inner recirculation loop, indicating that strain SZ persisted at high levels in the biostimulated zone throughout the biostimulation period. In August 2005, strain SZ cell numbers ranged from 10^5 to 10^6 per liter of groundwater in regions impacted by biostimulation (Fig. 2B). In contrast, the number of SZ cells was significantly lower in samples taken at the same time from areas not impacted by biostimulation (Fig. 2B). These observations indicate that strain SZ-like organisms were present at the Oak Ridge IFC site and, importantly, responded to biostimulation. The addition of ethanol and/or adjustment of other geochemical parameters (e.g., pH, nitrate removal [50, 51]) stimulated growth of strain SZ-like organisms. Although strain SZ cannot utilize ethanol as a direct electron donor, ethanol fermentation yields hydrogen and acetate, which are electron donors used by strain SZ (45). There is ample evidence that microbes indigenous to the Oak Ridge IFC site produce acetate when stimulated by ethanol: acetate accumulated (i) in ethanol-amended microcosms containing sediment from well FW104 (33), (ii) in an ethanol-amended soil column containing sediment from FW104 (12), and (iii) in portions of the area 3 biostimulated zone following ethanol addition (51). In a sulfate-reducing and U(VI)-reducing enrichment culture derived from the soil column and dominated by *Desulfovibrio*-like species, ethanol addition led to the accumulation of acetate in molar excess of the added amount, indicating homoacetogenenic activity (32). Hydrogen was produced intermittently. When the enrichment was transferred without sulfate, *Clostridium*-like bacteria became dominant while acetate and hydrogen accumulated. These observations indicate that several microbial processes produce acetate (and hydrogen) from ethanol in the Oak Ridge IFC site subsurface and that strain SZ could potentially partner with different types of native bacteria to reduce U(VI) and PCE. Previous work has demonstrated that *G. lovleyi* strain SZ reduces U(VI) and PCE simultaneously (45), and hence strain SZ is a likely contributor to in situ bioremediation at the mixed-waste Oak Ridge IFC site (48, 50).

The identification of key organisms contributing to contaminant detoxification [e.g., U(VI) reduction and reductive dechlorination] represents a targeted approach for bioremediation monitoring. Although comprehensive information about the microbial community composition and dynamics at bioremediation sites is desirable, the application of targeted mon-

itoring approaches to delineate and quantify the contributions of select organisms may produce tangible results in the short term. Monitoring key contributors, such as strain SZ-like organisms and other U(VI)-reducing bacteria (e.g., *Anaeromyxobacter* spp. and other metal reducers), to U(VI) bioreduction and reductive dechlorination at the Oak Ridge IFC site and other contaminated sites may provide sufficient information for productive implementation of bioremediation technologies.

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