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Detection and Quantification of *Dehalogenimonas* and "*Dehalococcoides*" Populations via PCR-Based Protocols Targeting 16S rRNA Genes▽†

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Members of the haloalkane dechlorinating genus *Dehalogenimonas* are distantly related to "*Dehalococcoides*" but share high homology in some variable regions of their 16S rRNA gene sequences. In this study, primers and PCR protocols intended to uniquely target *Dehalococcoides* were reevaluated, and primers and PCR protocols intended to uniquely target *Dehalogenimonas* were developed and tested. Use of the genus-specific primers revealed the presence of both bacterial groups in groundwater at a Louisiana Superfund site.

"*Dehalococcoides*" strains are the only bacteria presently known to reductively dehalogenate the carcinogen vinyl chloride (10–12, 17, 22), and DNA-based approaches have been widely applied to detect and quantify these bacteria in mixed cultures and environmental samples (1, 3, 4, 6, 7, 13, 15, 16, 20). As recently reported, *Dehalococcoides* strains are the closest previously cultured phylogenetic relatives of *Dehalogenimonas lykanthroporepellens* strains BL-DC-8 and BL-DC-9^T (18, 23). The newly isolated *Dehalogenimonas* strains, which can reductively dehalogenate a variety of polychlorinated alkanes (e.g., 1,2,3-trichloropropane and 1,2-dichloroethane) but not chlorinated ethenes (e.g., tetrachloroethene and vinyl chloride), however, are only distantly related to *Dehalococcoides*, with 90% identity in 16S rRNA gene sequences. Research reported here was aimed at (i) reevaluating PCR primers and protocols previously reported as allowing specific detection of *Dehalococcoides* 16S rRNA gene sequences in light of the 16S rRNA gene sequences of the recently isolated *Dehalogenimonas* strains and (ii) designing and testing PCR primers and protocols that allow detection and quantification of *Dehalogenimonas* strains.

Evaluation of *Dehalococcoides* 16S rRNA gene primer specificity. Twelve sets of previously published oligonucleotide primers targeting 16S rRNA gene sequences of *Dehalococcoides*, comprising 18 unique primer sequences, were evaluated (Table 1). Manual alignment of the *Dehalococcoides* primer sequences against the 16S rRNA gene sequences of *Dehalogenimonas lykanthroporepellens* strains BL-DC-8 and BL-DC-9^T (GenBank accession no. EU679418 and EU679419) revealed that primers Fp DHC 1, Fp DHC 774, and Rp DHC 806 (Table 1) exactly complement the corresponding binding regions in the 16S rRNA sequences of strains BL-DC-8 and BL-DC-9^T. Primer Fp DHC 385 contained a single mismatch. Among the 12 *Dehalococcoides* primer sets, set D (Table 1) had the lowest total number of mismatches, with only one base noncomplementary to the 16S rRNA gene sequences of *Dehalogenimonas* strains.

To experimentally test whether primers intended to target *Dehalococcoides* strains would amplify DNA from *Dehalogenimonas* strains, PCR was performed using the primer sets listed in Table 1, with strain BL-DC-9^T genomic DNA as a template. Details regarding genomic DNA preparation and construction of clone DNA from *Dehalogenimonas* and *Dehalococcoides* strains are presented as supplemental material. PCR was performed using 25-μl reaction volumes with the same concentrations of primers, Mg^{2+}, and deoxynucleoside triphosphates; thermal conditions; and cycle numbers as those specified in the original publications (Table 1). Hendrickson et al. (13) reported a range of 30 to 40 cycles, and the mid-range of 35 cycles was used in reactions testing primer sets A to G. AmpliTaq Gold DNA polymerase (Applied Biosystems, Foster City, CA) was used with activation at 95°C for 10 min. Following PCR, reaction products were electrophoresed in a 3% low-melting-temperature agarose gel (ISC BioExpress, Kaysville, UT), stained with ethidium bromide, and imaged.

PCR products of the sizes expected on the basis of 16S rRNA gene sequences of *Dehalogenimonas* and *Dehalococcoides* strains were observed for reactions employing the reportedly *Dehalococcoides*-specific A, D, and E primer sets, with *Dehalogenimonas* strain BL-DC-9^T genomic DNA as a template (see Fig. S1A in the supplemental material). Amplicons were also observed in additional PCRs, performed using plasmid DNA containing a partial 16S rRNA gene insert from strain BL-DC-9^T as a template, excluding the possibility that contamination of *Dehalococcoides* in strain BL-DC-9^T genomic DNA preparations might have resulted in PCR product formation. This demonstrates that some previously reported *Dehalococcoides*-specific primer sets in conjunction with their reported PCR thermal programs can amplify *Dehalogenimonas* sp. DNA and are therefore not specific to *Dehalococcoides*.

Additional experiments performed using the same primers and reagent concentrations as those mentioned above but at successively higher annealing temperatures revealed that an-

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TABLE 1. *Dehalococcoides*-specific 16S rRNA gene primer sets evaluated in this study

Primer set	Primer	Primer sequence[a] (5′–3′)	Reference	Reported T_a (°C)[b]	Amplicon size (bp)	No. of mismatches[c] (bp)	False positive[d]	Revised T_a (°C)[e]
A	Fp DHC 1	GAT GAA CGC TAG CGG CG	13	55	692	0	+	62
	Rp DHC 692	TCA GT**G** **ACA** ACC TAG AAA AC				5		
B	Fp DHC 1	GAT GAA CGC TAG CGG CG	13	55	1,212	0	−	NA
	Rp DHC 1212	GGA TTA GCT CC**A** G**TT** CAC **ACT** G				4		
C	Fp DHC 1	GAT GAA CGC TAG CGG CG	13	55	1,377	0	−	NA
	Rp DHC 1377	GGT T**GG** C**AC** ATC GAC TTC A**A**				4		
D	Fp DHC 385	GGG TTG TAA ACC TCT TTT C**AC**	13	55	421	1	+	68
	Rp DHC 806	GTT AGC TTC GGC ACA GAG AG				0		
E	Fp DHC 587	GGA CT**A** GAG T**AC** AGC AGG AGA AAA C	13	55	503	3	+	66
	Rp DHC 1090	GGC AGT **C**TC GCT AGA **A**AA T				2		
F	Fp DHC 774	GGG AGT ATC GAC CCT CTC	13	55	438	0	−	NA
	Rp DHC 1212	GGA TTA GCT CC**A** G**TT** CAC **ACT** G				4		
G	Fp DHC 946	AGT GAA CCG AAA GGG **AAA**	13	55	266	2	−	NA
	Rp DHC 1212	GGA TTA GCT CC**A** G**TT** CAC **ACT** G				4		
H	728f	AAG GCG GTT TTC TAG G**TT** **GTC** AC	15	58	444	5	−	NA
	1172r	CGT TT**C** GCG **G**GG CAG **TCT**				3		
I	1f	GAT GAA CGC TAG CGG CG	6	59	258	0	−	NA
	259r	CAG ACC AGC TAC CGA TC**G** **AA**				2		
J	582f	CTG TTG GAC TAG AGT **ACA** GC	6	59	108	3	−	NA
	728r	GT**G** **ACA** ACC TAG AAA ACC GCC TT				5		
K	DeF	GCA A**TT** **A**AG ATA GTG GC	3	55	1,373	3	−	NA
	DeR	ACT TCG TCC CAA T**TA** **CC**				2		
L	DHE-for	AAG GCG GTT TTC TAG G**TT**	5	58	443	2	−	NA
	DHE-rev	CGT TT**C** GCG **G**GG CAG **TCT**				3		

[a] Mismatches between the *Dehalococcoides* primer sequences and the 16S rRNA gene sequences of *Dehalogenimonas lykanthroporepellens* strains BL-DC-8 and BL-DC-9^T are indicated by bolded text.
[b] Annealing temperature (T_a) reported for the PCR or denaturing gradient gel electrophoresis-PCR thermal protocol.
[c] Number of nucleotide mismatches in each primer relative to strain BL-DC-9^T.
[d] "+" indicates that the primer set resulted in amplification when genomic DNA from *Dehalogenimonas* strains BL-DC-8 and BL-DC-9^T or plasmid DNA containing partial 16S rRNA inserts from these strains was used as the template. "−" indicates that amplification was not observed.
[e] PCR annealing temperature (T_a) that did not lead to amplification of *Dehalogenimonas* strains BL-DC-8 and BL-DC-9^T. NA, not applicable.

nealing temperatures of 62, 68, and 66°C for *Dehalococcoides* primer sets A, D, and E, respectively, did not result in detectable PCR amplification of *Dehalogenimonas* strain BL-DC-9^T DNA but did produce amplicons in reactions using plasmid DNA from *Dehalococcoides* clone DHC-4 as a template (see Fig. S1B in the supplemental material). This demonstrated that the primer sets can allow specific detection of *Dehalococcoides* 16S rRNA genes if annealing temperatures are sufficiently high.

It appears that the amplification of *Dehalogenimonas* 16S rRNA genes by use of *Dehalococcoides* primers was influenced more by the locations of the mismatches than by the total number of mismatches in the primer/DNA sequence. For example, primer sets A and E both have combined totals of five base mismatches relative to the sequence of strain BL-DC-9^T. The mismatches, however, are not located at the priming ends. This allowed amplification of *Dehalogenimonas* 16S rRNA genes when the relatively low annealing temperature of 55°C was employed. In contrast, although primer set I has only two mismatches relative to the sequence of *Dehalogenimonas* strain BL-DC-9^T, no amplification was observed with annealing at 59°C. This likely resulted because the two mismatches are located at the priming ends (Table 1).

16S rRNA gene primers targeting *Dehalogenimonas* sp. Details of the methodology used to design primers intended to target 16S rRNA gene sequences unique to *Dehalogenimonas* are given in the supplemental material. Thirteen primer combinations (Table 2) were experimentally tested. In initial tests for verification of primer function, genomic DNA from strain BL-DC-9^T was used as a template in PCR. Each reaction mixture contained 0.5 μM each primer, 2.5 mM $MgCl_2$, 100 μM each deoxynucleoside triphosphate, and 2 U of AmpliTaq Gold in 1× PCR gold buffer (Applied Biosystems). The thermal program included initial denaturation at 95°C for 10 min; followed by 35 cycles of 1.0 min at 94°C, 45 s at 63°C, and 1.0 min at 72°C; and a final extension step of 10 min at 72°C. All 13 primer combinations intended to specifically target 16S rRNA gene sequences of *Dehalogenimonas* strains (Table 2)

TABLE 2. Sequences of *Dehalogenimonas*-specific 16S rRNA gene primers employed in this study

Primer set	Primer	Positions[a]	Primer sequence[b] (5′–3′)	Amplicon size (bp)	No. of mismatches[c] (bp)
M	BL-DC-57f	57–84	GCA AGT CGA ACG GTC TCT CGC	1,330	3
	BL-DC-1410r	1421–1442	AGG TGT TAC CAA CTT TCA TGA C		3
N	BL-DC-57f	57–84	GCA AGT CGA ACG GTC TCT CGC	1,271	3
	BL-DC-1351r	1362–1383	AAC GCG CTA TGC TGA CAC GCG T		6 or 7
O	BL-DC-117f	117–140	GTA ATA GGT AAG TAA CCT GCC CTT	911	6
	BL-DC-1020r	1026–1052	ATA GCT CCT GAC TTG ACA GGT GGA TC		6 or 7
P	BL-DC-142f	143–170	GTG GGG GAT AAC ACT TCG AAA GAA GTG C	661	12
	BL-DC-796r	799–827	ACC CAG TGT TTA GGG CGT GGA CTA CCA GG		6
Q	BL-DC-142f	143–170	GTG GGG GAT AAC ACT TCG AAA GAA GTG C	885	12
	BL-DC-1020r	1026–1052	ATA GCT CCT GAC TTG ACA GGT GGA TC		6 or 7
R	BL-DC-142f	143–170	GTG GGG GAT AAC ACT TCG AAA GAA GTG C	1,093	12
	BL-DC-1243r	1253–1275	CCG GTG GCA ACC CAT TGT ACC GC		7
S	BL-DC-183f	184–210	GGT GCT CTT TCA CAA GGA AGA GTA CTG	620	13 or 14
	BL-DC-796r	799–827	ACC CAG TGT TTA GGG CGT GGA CTA CCA GG		6
T	BL-DC-610f	613–642	TCT CCC GGC TCA ACT GGG AGG GGT CAT CTG	439	8
	BL-DC-1020r	1026–1052	ATA GCT CCT GAC TTG ACA GGT GGA TC		6 or 7
U	BL-DC-610f	613–640	TCT CCC GGC TCA ACT GGG AGG GGT CAT CTG	647	8
	BL-DC-1243r	1253–1275	CCG GTG GCA ACC CAT TGT ACC GC		7
V	BL-DC-727f	730–751	GAA GGC GGT TTT CTA GGC CAW A	322	4
	BL-DC-1020r	1026–1052	ATA GCT CCT GAC TTG ACA GGT GGA TC		6 or 7
W	BL-DC-727f	730–751	GAA GGC GGT TTT CTA GGC CAW A	636	4
	BL-DC-1351r	1362–1383	AAC GCG CTA TGC TGA CAC GCG T		6 or 7
X	BL-DC-631f	634–663	GGT CAT CTG ATA CTG TTG GAC TTG AGT ATG	194	7
	BL-DC-796r	799–827	ACC CAG TGT TTA GGG CGT GGA CTA CCA GG		6
Y	BL-DC-727f	730–751	GAA GGC GGT TTT CTA GGC CAW A	278	4
	BL-DC-982r	986–1007	TCT AAC ATG TCA AGC CCT GGT G		7 or 8

[a] Base position according to *Escherichia coli* 16S rRNA gene sequence coordinates (GenBank accession no. E05133).
[b] W equals A or T.
[c] Number of noncomplementary nucleotide positions in alignment with *Dehalococcoides* strains CBDB1, 195, VS, BAV1, and FL2.

produced bands with the expected sizes in PCRs with strain BL-DC-9^T genomic DNA used as the template (see Fig. S2A in the supplemental material). No PCR products were observed for reactions with plasmid DNA from *Dehalococcoides* clone DHC-4 used as the template under identical PCR conditions (data not shown), indicating that the primer sets intended to target *Dehalogenimonas* strains did not amplify DNA from *Dehalococcoides* strains.

Environmental samples. Groundwater was collected from eight wells (identification no. W-0627-2, W-0721-1, W-0726-4, W-0820-1, W-0822-3, W-0823-2, W-0825-1, and W-0828-1) at the PetroProcessors of Louisiana, Inc. Superfund site, where contaminants remain in the subsurface as dense nonaqueous-phase liquid (DNAPL), and high concentrations of chlorinated solvents, including 1,1,2-trichloroethane, 1,2-dichloroethane, 1,2-dichloropropane, and vinyl chloride, are present in the aqueous phase (1). All eight groundwater samples, collected in sterile 1.0-liter glass bottles, were visually observed to contain DNAPL. After transport to the laboratory on ice (approximately 1 h), a 30-ml volume of groundwater from each well was mixed with 10 ml TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0) and centrifuged for 10 min at 3,200 × *g*, the supernatant was decanted, and then DNA was extracted and purified as described previously (1).

DNA extracted from one groundwater sample (W-0823-2) served as the template in separate PCRs using all 13 primer combinations listed in Table 2. All 13 primer sets yielded PCR products of the sizes expected on the basis of the 16S rRNA gene sequences of strains BL-DC-8 and BL-DC-9^T (see Fig. S2B in the supplemental material). Sequencing determined that all 13 PCR products were identical to the 16S rRNA gene sequences of *Dehalogenimonas* strains BL-DC-8 and BL-DC-9^T, providing an indication of primer specificity.

DNA extracts from all eight groundwater samples were used as a template in separate PCRs with primer set X (Table 2), targeting *Dehalogenimonas* spp., and primer set J (Table 1), targeting *Dehalococcoides* spp., as described above. The annealing temperatures were 63°C and 56°C for reactions using

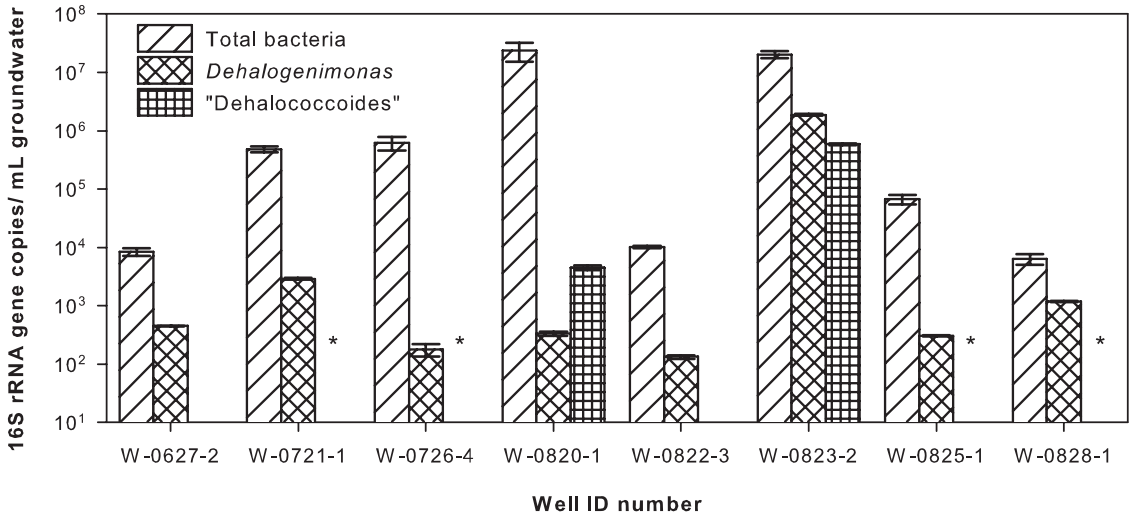

FIG. 1. qPCR quantification of total bacteria and *Dehalogenimonas* and *Dehalococcoides* 16S rRNA gene copies in groundwater samples from the DNAPL source zone at the PetroProcessors of Louisiana, Inc., Superfund site. Asterisks denote the quantification limit for samples where the target was detected but at a level below the linear range of the calibration curve. Error bars represent standard deviations of results from triplicate measurements for each well sampled. ID, identification.

primer sets X and J, respectively. For cases where no amplification products were detected, nested PCR was employed with initial amplification using universal bacterial primers 530f/900r (14) with the PCR reagent composition and conditions described above but with an annealing temperature of 56°C. PCR products purified using an UltraClean PCR cleanup kit (MoBio) were then used as a template in a second reaction, using primer set X or J.

PCR products corresponding to *Dehalogenimonas* 16S rRNA gene sequences were detected in seven groundwater samples, and *Dehalococcoides* 16S rRNA gene sequences were detected in six samples (see Fig. S3 in the supplemental material). Sequencing of PCR products generated using *Dehalococcoides* primer set J revealed that amplicons shared 100% identity with sequences of *Dehalococcoides* strains BAV1, FL2, CBDB1, and 195 (GenBank accession no. AY165308, AF357918, AF230641, and AF004928, respectively), which are identical over the region amplified. The amplicons generated using *Dehalogenimonas*-targeting primer set X with groundwater DNA extracts from six wells were identical to the 16S rRNA gene sequence of strain BL-DC-9^T. The PCR products from the other well had three nucleotide mismatches relative to strain BL-DC-9^T.

qPCR. *Dehalogenimonas*-targeting primer set X (Table 2) and *Dehalococcoides*-targeting primer set J (Table 1) were used in quantitative real-time PCR (qPCR) to evaluate concentrations of putative dehalogenating bacteria in groundwater samples. To allow calculation of abundance relative to total bacterial populations, universal bacterial primers Bac1055YF/Bac1392R (20) were employed to quantify total bacterial 16S rRNA gene copies. Details regarding qPCR experimental methods and data analysis are provided as supplemental material.

In qPCR, amplicons corresponding to 16S rRNA gene sequences of *Dehalogenimonas* strains were detected in all eight groundwater samples analyzed, with concentrations ranging from $(1.33 \pm 0.09) \times 10^2$ to $(1.88 \pm 0.07) \times 10^6$ copies/ml (Fig. 1). *Dehalococcoides* 16S rRNA genes were detected in six groundwater samples; however, concentrations were below the linear range of the calibration curve for all but two samples. The concentrations ranged from $<2.8 \times 10^3$ copies/ml (i.e., below linear range of the calibration curve) to $(5.84 \pm 0.20) \times 10^5$/ml. The highest concentrations of both *Dehalogenimonas* and *Dehalococcoides* strains were in the sample from well W-0823-2, with concentrations of $(1.88 \pm 0.07) \times 10^6$ and $(5.84 \pm 0.20) \times 10^5$ 16S rRNA gene copies/ml, respectively. PCR amplicons generated using primer sets X and J each produced a single melting curve peak, further indicating primer specificity in analysis of these environmental samples (data not shown). Total bacterial 16S rRNA gene copies in the groundwater samples ranged from $(8.40 \pm 1.25) \times 10^3$ to $(2.38 \pm 0.86) \times 10^7$ copies/ml (Fig. 1). The upper end of this range is consistent with the observation of $>3 \times 10^7$ cells/ml groundwater in direct microscopic counts in a previous study of the DNAPL source zone at this site (1).

For well W-0828-1, gene copy numbers determined using *Dehalogenimonas*-targeting primers were equal to 18.6% of total bacterial 16S rRNA gene copies. Such high relative abundance of dehalogenating bacteria has previously been reported only for enrichment cultures provided with a readily available supply of electron donors (e.g., H_2) and other favorable growth conditions (1, 6, 9, 19). In other wells, *Dehalogenimonas* 16S rRNA gene copy numbers represented smaller percentages of the total bacterial 16S rRNA gene copies, 0.0014 to 9.23%. *Dehalococcoides* 16S rRNA gene copies in the two samples falling within the linear range of the qPCR calibration curve comprised 0.02 and 2.87% of total bacterial 16S rRNA gene copies.

This study demonstrated the presence of *Dehalogenimonas* strains with *Dehalococcoides* strains in groundwater from a DNAPL source zone for the first time. Detection of both bacterial groups, neither of which is known to grow in the ab-

sence of chlorinated solvents, supports the notions that (i) dechlorinating bacteria may reside in close proximity to DNAPLs (2, 5, 21, 24) and (ii) dechlorination may involve multiple dehalogenating populations (8). It also expands the genera implicated in multispecies dechlorinating populations to include *Dehalogenimonas*. Primers reported here for *Dehalogenimonas* and protocols that were found here to allow unique detection of *Dehalococcoides* will prove useful in future studies for detection and quantification of these taxa.

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