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A Novel Reductive Dehalogenase, Identified in a Contaminated Groundwater Enrichment Culture and in *Desulfitobacterium dichloroeliminans* Strain DCA1, Is Linked to Dehalogenation of 1,2-Dichloroethane▽

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A mixed culture dechlorinating 1,2-dichloroethane (1,2-DCA) to ethene was enriched from groundwater that had been subjected to long-term contamination. In the metagenome of the enrichment, a 7-kb reductive dehalogenase (RD) gene cluster sequence was detected by inverse and direct PCR. The RD gene cluster had four open reading frames (ORF) showing 99% nucleotide identity with *pceB*, *pceC*, *pceT*, and *orf1* of *Dehalobacter restrictus* strain DSMZ 9455^T, a bacterium able to dechlorinate chlorinated ethenes. However, *dcaA*, the ORF encoding the catalytic subunit, showed only 94% nucleotide and 90% amino acid identity with *pceA* of strain DSMZ 9455^T. Fifty-three percent of the amino acid differences were localized in two defined regions of the predicted protein. Exposure of the culture to 1,2-DCA and lactate increased the *dcaA* gene copy number by 2 log units, and under these conditions the *dcaA* and *dcaB* genes were actively transcribed. A very similar RD gene cluster with 98% identity in the *dcaA* gene sequence was identified in *Desulfitobacterium dichloroeliminans* strain DCA1, the only known isolate that selectively dechlorinates 1,2-DCA but not chlorinated ethenes. The *dcaA* gene of strain DCA1 possesses the same amino acid motifs as the new *dcaA* gene. Southern hybridization using total genomic DNA of strain DCA1 with *dcaA* gene-specific and *dcaB*- and *pceB*-targeting probes indicated the presence of two identical or highly similar dehalogenase gene clusters. In conclusion, these data suggest that the newly described RDs are specifically adapted to 1,2-DCA dechlorination.

Chlorinated alkanes are prevailing groundwater contaminants in many industrialized countries (www.epa.gov/enviro/html/tris/ez.html), and they cause serious environmental problems (14). Among these, 1,2-dichloroethane (1,2-DCA) represents one of the world's most important toxic C_2 chlorinated aquifer pollutants. From 1987 to 1993, over 209 tons of 1,2-DCA were released into groundwater (www.epa.gov/enviro/html/tris/ez.html). Enhancement of natural attenuation processes can play a major role in achieving remediation, where applicable (17, 30), and often bioremediation alone or in combination with physical treatment of the most contaminated areas could represent the most convenient solution. The main catalyzers of the bioremediation processes are microorganisms that can dehalogenate and/or mineralize the toxic molecules with their enzymatic systems (12, 40).

A metabolic process of particular interest is based on dehalorespiration, a reaction that couples reductive dehalogenation with energy conservation (11, 35, 40). Dehalorespiration is one of the key processes for the remediation of polluted groundwaters (20, 24, 25). The key catalysts in dehalorespiration are reductive dehalogenases (RDs), membrane-associated enzymes with low levels of nucleotide identity but with some common traits, such as two iron-sulfur clusters as prosthetic groups, a twin-arginine translocation signal peptide (TAT system), and corrinoid cofactors (22). Recently, it has been reported that RDs (e.g., the cluster of *pceABC* and *pceT* of *Desulfitobacterium hafniense* TCE-1 or Y-51) can be part of catabolic transposons that can mediate RD mobilization within replicons and between bacteria (10, 22, 34).

The best-characterized RDs for halogenated aliphatics are those specific for chloroalkenes, such as the RDs from some *Dehalococcoides* spp. (see references 18 and 45 and references therein), the RDs for tetrachloroethene (PCE) from *Dehalobacter restrictus* strain DSMZ 9455^T (23), *Desulfitobacterium* sp. strain Y51 (41), and *Desulfitobacterium hafniense* strain PCE-S (23), and the RD for trichloroethene and PCE from *Desulfitobacterium hafniense* strain TCE1 (23). Recently, De Wildeman et al. (6) isolated *Desulfitobacterium dichloroeliminans* strain DCA1, which can efficiently dechlorinate 1,2-DCA by using hydrogen as an electron donor; however, no specific RDs for 1,2-DCA have yet been characterized.

A 1,2-DCA-specific RD would be of particular interest because it can support dichloroelimination when the two chlorine atoms of 1,2-DCA are removed, converting the contaminant into ethene with no toxic chlorinated intermediates such as vinyl chloride. This reaction is thermodynamically favorable, since it requires only one H_2 molecule for the removal of two chlorine substituents (8, 16, 26, 40).

To our knowledge, this is the first study identifying a 1,2-DCA-specific RD gene cluster in the metagenome of a 1,2-DCA deha-

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lorespiring enrichment culture from a contaminated aquifer and in *D. dichloroeliminans* strain DCA1. Real-time quantitative PCR (qPCR), reverse transcription-PCR (RT-PCR), Southern hybridization, and long-range PCR were used to show the involvement of the RD in the dechlorination of 1,2-DCA. Comparative sequence analysis of the RD catalytic subunit (DcaA) of the enrichment culture and of *D. dichloroeliminans* strain DCA1 with previously described RDs highlighted specific sequence differences and signature motifs, suggesting that the two new 1,2-DCA-specific RDs may represent enzymes specifically adapted to 1,2-DCA reductive dechlorination.

MATERIALS AND METHODS

Establishment of enrichment culture 6VS and cultivation of *D. dichloroeliminans* strain DCA1. Anaerobic microcosms were prepared in 2003 with groundwater from an aquifer in Italy that had been contaminated by 1,2-DCA for more than 30 years (25). The groundwater was contaminated with 1,2-DCA at a very high concentration (up to 9 mM) but not with other chlorinated ethanes or ethenes. The microcosms were prepared in an anaerobic box in an atmosphere of N_2 and fed with 5 mM sodium lactate. The microbial community from one of these microcosms was used to inoculate a glass column (internal diameter, 4 cm; height, 12 cm) filled with active carbon withdrawn from an on-site "pump and treat" system coupled with an active carbon filter used in the past for the removal of 1,2-DCA from the groundwater. The total internal volume in the column after active carbon addition was 50 ml. The glass column, set up to maintain a storage culture, was filled with half a volume of microcosm culture and half a volume of a sterile prereduced water solution amended with 1 mM cysteine, 50 mg liter^{-1} vitamin B12, 0.5 mM HEPES–NaOH (pH 7) [4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid solution], 0.05% (wt/vol) yeast extract, and 50 μM 2-methyl-1,4-naphtoquinone sodium bisulfite (vitamin K precursor). This solution was also amended with a 1:200 dilution of a trace-element stock solution and a supplementary salt solution as described by Marzorati et al. (25). Every 60 days 10 ml of the liquid volume in the column was withdrawn under a nitrogen flux and replaced with a fresh sterile solution and 8 mM 1,2-DCA.

After one year from the establishment of the storage culture in the active carbon column, a suspended culture named 6VS was prepared by mixing 25 ml of the active carbon culture with an equal volume of the sterile solution described above in a 60 ml screw-top glass vial with Teflon septa. The culture was supplemented with 1,2-DCA and lactate to final concentrations of 8 mM and 5 mM, respectively, and incubated in $N_2/CO_2/H_2$ (80%/15%/5%) at room temperature (average temperature, 22°C). After 40 days of incubation, when dechlorination ceased, 50% of the culture was replaced with fresh sterile anaerobic medium, 1,2-DCA, and lactate as described above. The 6VS culture was transferred six times before analysis of the bacterial composition was performed using PCR-denaturing gradient gel electrophoresis (PCR-DGGE) and sequencing of the 16S rRNA genes.

The *D. dichloroeliminans* strain DCA1 culture was set up according to the procedures developed by De Wildeman et al. (6).

Analytical methods. The concentrations of 1,2-DCA, vinyl chloride, and other possible degradation products in the 6VS culture were analyzed by headspace gas chromatography using a 7694 Agilent gas chromatograph equipped with a flame ionization detector set at 200°C on a DB624 column (J&W Scientific, Folsom, CA) at a constant oven temperature of 80°C. The limit of detection for 1,2-DCA was about 1 to 2 μg liter^{-1}.

The chloride concentrations were measured on a PCspectro photometer (Lovibond, Dortmund, Germany) by utilization of method 180 chloride concentration (Tintometer GmbH, Dortmund, Germany) for chloride determination in the range of 5 to 60 mg liter^{-1}.

DNA extraction from culture 6VS and *D. dichloroeliminans* strain DCA1 and PCR-DGGE analysis. DNA from 6VS culture and *D. dichloroeliminans* strain DCA1 culture was extracted from a 1.5 ml sample by treatment with lysozyme, proteinase K, cetyltrimethylammonium bromide, and sodium dodecyl sulfate as described by Ausubel et al. (2).

DGGE analysis was conducted using 16S rRNA gene fragments amplified with primers GC-EUB Fw and EUB Rv as described elsewhere (15). A 7% polyacrylamide gel was prepared with a gradient maker (Bio-Rad, Milan, Italy) according to the manufacturer's guidelines, with a denaturant gradient of 25% (top) to 50% (bottom), where 100% denaturation is considered to be represented by the presence of 7 M urea and 40% formamide, according to the method of Muyzer et al. (27). Individual bands were cut from the gel and sequenced on a Mega BACE capillary electrophoresis instrument (Amersham-Pharmacia, Milan, Italy) following the instructions of the manufacturer. To obtain longer 16S rRNA sequences, the original sequences obtained by DGGE band elution and sequencing were analyzed to detect species-specific DNA stretches on which new primers were designed. These new primers, coupled to bacterial universal primers, were used for PCR amplification reactions and extension of the original 16S rRNA sequence fragment by using the original metagenomic DNA as the template. In particular, the following reverse primers were used to extend DGGE band sequences: B1rev (5′-TGTACCGACCATTG TATCA-3′) and B2rev (5′-TGTACCGGCCATTGTATTA-3′) coupled to primer 357F (5′-CCCTACGGGAGGCAGCAG-3′) (36).

16S rRNA quantitative real-time PCR. Quantitative real-time PCR analysis of the genera *Dehalobacter* and *Desulfitobacterium* was performed using a GeneAmp 5700 instrument (Applied Biosystems) and the following primers: 933F (5′-GCACAAGCGGTGGAGCATGTGG-3′) and 1178R (5′-TATCTAG AGTGCTC(AG)ACCT-3′) specific for *Desulfitobacterium* spp. or 1366R (5′-ACAATCCGAACTGAGAACG-3′) specific for *Dehalobacter* spp. The concentration of each metagenomic DNA sample was estimated using a Nanodrop instrument (Celbio, Milan, Italy), and the equivalent of 10 and 100 pg DNA (a double reaction was used for each sample) was used in a 50 μl reaction with DyNAmoTM HS SYBR green qPCR (Celbio, Milan, Italy) according to the instructions of the manufacturer. An initial hot start at 50°C for 2 min and denaturation at 94°C for 15 min were followed by 35 cycles consisting of denaturation at 94°C for 10 s, annealing at 53°C (*Desulfitobacterium* spp.) or 55°C (*Dehalobacter* spp.) for 30 s, and extension at 72°C for 30 s. A final step for melting-curve analysis from 72 to 95°C, with measurement of fluorescence every 0.5°C, was added. PCR products for standard curve were cloned using a pGEM-T Easy vector cloning kit (Promega). Standard curves had an average correlation coefficient of 0.998 and a slope of −3.624 for *Desulfitobacterium*-specific qPCR and an average correlation coefficient of 0.997 and a slope of −3.488 for *Dehalobacter*-specific qPCR.

PCR amplification, cloning, and sequencing of reductive dehalogenase. To identify potential catabolic genes involved in reductive dehalogenation, PCR amplification with degenerated primers (ceRD2Sf, ceRD2Lf, RD7r) was performed on DNA extracted from 6VS culture according to the method of Regeard et al. (33) as reported in Marzorati et al. (25). Positive PCR products were cloned using a pMOS Blue cloning kit (Amersham-Pharmacia) according to the manufacturer's instructions. A direct PCR assay was performed on white colonies to amplify the insert by using primers T7 and U19 (32). PCR products were sequenced with T7 primer by using ABI Prism BigDye Terminator cycle sequencing (Applied Biosystems, Milan, Italy) and an ABI 310 automated sequencer (Applied Biosystems) (25). The resulting sequences were compared with the sequence database at the National Center for Biotechnology Information (NCBI) by use of BLAST (1).

Inverse and direct PCR. All the primers used for inverse PCR, direct PCR, and sequencing of the RD gene cluster from the metagenomic DNA of 6VS culture and total DNA of *D. dichloroeliminans* strain DCA1 are summarized in Table 1. Starting from the initial partial RD gene fragment (25), the complete sequence of an RD gene cluster was obtained by a combination of one inverse PCR (43) and four different standard PCRs (Fig. 1). The inverse-PCR approach was used initially to obtain the flanking regions of the initial gene fragment. The remaining parts of the gene cluster were obtained by standard PCR walking (Table 1).

Metagenomic DNA (250 ng) from 6VS culture was digested with EcoRI restriction enzyme (Promega, Milan, Italy) for 15 h at 37°C. Five units of T4 DNA ligase (Promega), 5 μl of the 10× ligation buffer (Promega), and MilliQ water for a final volume of 50 μl were then added to the digested genomic DNA. The ligation mixture was incubated for 1 h at 22°C and then at 10 min at 65°C to inactivate the enzyme. A 3-μl volume of the ligation mix was then used as a template for PCR using the following components: 1× PCR buffer (Amersham-Pharmacia), 1.5 mM $MgCl_2$, 0.3 mM (each) deoxynucleoside triphosphates (dNTPs), 0.5 μM each primer, and 1 U of *Taq* polymerase in a final volume of 50 μl. Initial denaturation at 94°C for 4 min was followed by 30 cycles consisting of denaturation at 94°C for 45 s, annealing at 58°C for 1 min, and extension at 72°C for 2 min 15 s. A final extension at 72°C for 7 min was added.

Direct PCR experiments were conducted with the following reaction mixture: 1× PCR buffer (Amersham-Pharmacia), 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.3 μM each primer, and 1 U of *Taq* polymerase in a final volume of 50 μl. Initial denaturation at 94°C for 4 min was followed by 30 cycles consisting of denaturation at 94°C for 45 s, annealing at 52°C for 1 min, and extension at 72°C for about 1 min for every 1,000 bp of expected product. A final extension at 72°C for 7 min was added.

Long-range PCR experiments to amplify the gene cluster were conducted

TABLE 1. Primers designed in this study and used for inverse PCR, direct PCR, and sequencing of environmental RD-54 and RD-DCA1 of *D. dichloroeliminans* strain DCA1[a]

Primer	DNA(s) used for PCRs	Targeted gene or spacer[b]	Orientation[c]	5′–3′ sequence	Nucleotide position(s)[d]
DHL-FOR	6VS	*dcaA*	For	AATTCGGGGTACGCGAGT	2771
DHL-REV	6VS, DCA1	*dcaA*	Rev	CAGGCTCATTAGCTATTTCA	2097–1991
DHL-RevA	6VS	*dcaA*	Rev	TACTTTGCATCCACCTTG	1696
PceT-F	6VS	*dcaA*	For	GTATGAATTTGATGAAGAAG	5645
DHL-orf-Rev	6VS	*dcaA*	Rev	AGGAGAGAACCTTAATCG	7460
DHL-fin-Rev	6VS	*dcaA*	Rev	GGATTTGTTCCTCATCCT	6824
DHL F1	6VS	*dcaA*	For	GGACCTCGTTGGACTCC	1967
DHL F2	6VS	*dcaA*	For	GTTAAAAAGGCAGCCTGTT	2220
DHL R1	6VS	*dcaA*	Rev	GGCAAATCCCATGGCATTA	2350
DHL R2	6VS	*dcaA*	Rev	GTAAACTTTCCCCGTCGC	2528
RDdca1R1	DCA1	*dcaA*	Rev	TCCTCCTGTTGATTTCGC	1415
RDdca1F1	DCA1	*dcaA*	For	AATACCTTGTTGGATGACG	1843
RDdca1R2	DCA1	*dcaA*	Rev	TTGGTTTGAAACCCGCATA	2311
DH3F	6VS	*dcaA*	For	ATTGGGAGAAGCATGCAGGT	2420
DH3R	6VS	*dcaA*	Rev	GACCACCGTTATAGGCCCAGA	2946
Dca1F	DCA1	*dcaA*	For	CAGGCAAGAAAGATACGG	1709
DHL-ForA	6VS, DCA1	*dcaB*	For	TCGGAGCGTGAATACCA	3429–3306
DcaB rev	6VS, DCA1	*dcaB*	Rev	TGGTATTCACGCTCCGA	3429–3306
Dca1BF	DCA1	*dcaB*	For	TATATGATGTGCTGATTTGG	3136
Dca1BR	DCA1	*dcaB*	Rev	GTAAGTAATAATCGCAGGAA	3367
DcaC-F2	6VS, DCA1	*dcaC*	For	ATTCTTCTGTCCGGTAGGAT	4808–4702
PceC-rev2	6VS, DCA1	*dcaC*	Rev	TACCTCCTCATTTCGCC	4852–4746
PceT-rev2	6VS, DCA1	*dcaT*	Rev	GATTAACTTGCCAAATTGAT	6021–5922
dcaTfwDCA1	DCA1	*dcaT*	For	TCTTGAAAAGCGAATTAACG	5783
DCA1dcaTr	DCA1	*dcaT*	Rev	GATTAACTTGCCAAATTGATT	5907
DCA1ups	DCA1	*tpnA*	For	ATGCAAAGCTAGGTGCTG	0
tpnDCA1rev	DCA1	*tpnA*	Rev	TACAGTGTCCCCATCCG	86
DCA1dwnR	DCA1	*tpnA*	Rev	AGAGACTGGTGTAGGTTG	498
PceAFor1	6VS, DCA1	*tpnA-dcaA* spacer	For	ACGTGCAATTATTATTAAGG	1485–1379
Dehalo-PCE	6VS	Upstream *tpnA*	For	ATAATGACTCAACTTTCGAA	0
DHL-ups-Mid	6VS	Upstream *tpnA*	For	TGTGTAGGAGTTACGACA	663

[a] The total DNAs used for the PCRs were from culture 6VS (6VS) and from *D. dichloroeliminans* strain DCA1 (DCA1).
[b] Please refer to Fig. 1 and the corresponding figure legend for the gene positions and fragments originating from PCRs.
[c] For, forward; Rev, reverse.
[d] The nucleotide position refers to the position of the primer in the complete RD sequence of the samples with which they have been used. Where there are two numbers, the first refers to RD-54 and the second to RD-DCA1.

using the following reaction mixture: 1× PCR buffer (Amersham-Pharmacia), 1.5 mM $MgCl_2$, 0.5 mM dNTPs, 0.6 μM each primer, and 2.5 U of *Taq* polymerase in a final volume of 50 μl. Initial denaturation at 94°C for 4 min was followed by 31 cycles consisting of denaturation at 94°C for 50 s, annealing at 52°C for 50 s, and extension at 72°C for 8 min. A final extension at 72°C for 7 min was added. Primers PceAFor1 and PceC-rev2 were used on metagenomic DNA of culture 6VS, while primers RDdca1F1 and PceT-rev2 were used on DNA of strain DCA1.

The percentage of residue identity among proteins was calculated using Vector NTI software (21), and the amino acid sequences were subjected to neighbor-joining analysis to assess the phylogenetic relationship. The ratio of nonsynonymous (dN) to synonymous (dS) nucleotide changes in the amino acid sequences was calculated according to the method of Nei and Gojobori (28) by using Mega software version 3.1 (19).

Specific PCR to identify *dcaAB* genes and quantitative PCR. Using primers DHL F1, DHL F2, DHL R1, and DHL R2 specific for the *dcaA* gene and DcaB rev specific for the *dcaB* gene (Table 1), a direct PCR to identify the presence of the RD was developed with the following reaction mixture: 1× PCR buffer (Amersham), 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.6 μM each primer, and 1 U of *Taq* polymerase in a final volume of 25 μl. Initial denaturation at 94°C for 3 min

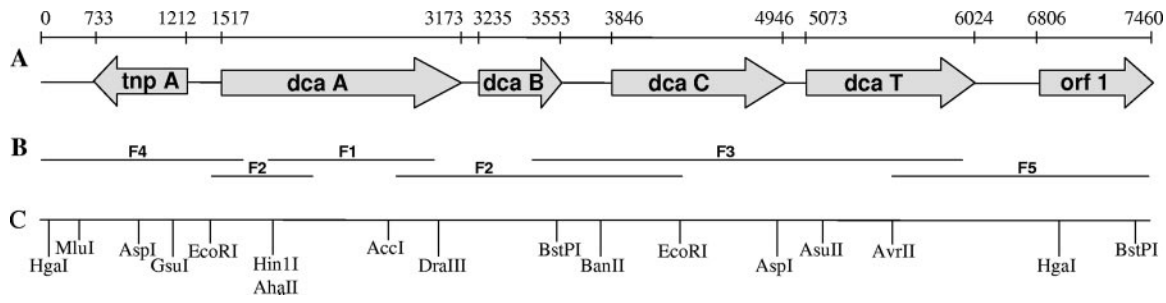

FIG. 1. Map of the RD-54 gene cluster obtained from the metagenome of culture 6VS by direct and inverse PCR. A) Complete map of RD-54 with the relative initial and final positions of each gene. B) Map of the direct and inverse PCR fragments obtained from the total metagenome of culture 6VS. The different PCR fragments from F1 to F5 were generated with the following primer pairs as referred to Table 1 (fragment sizes are shown in parentheses): F1, ceRD2Lf/RD7rev (1,036 bp); F2 (inverse PCR), DHL-FOR and DHL-REV (2,300 bp); F3, DHL-for and PceT-rev2 (2,566 bp); F4, Dehalo-PCE and DHL-RevA (1,678 bp); and F5, PceT-F and DHL-orf-Rev (1,815 bp). C) Partial restriction map of RD-54.

was followed by 31 cycles consisting of denaturation at 94°C for 30 s, annealing at 54°C for 1 min, and extension at 72°C for 1 min 15 s. A final extension at 72°C for 7 min was added. The same protocol (with an extension time of 2 min) was used for coupling primers PceAFor1 and DcaB rev to amplify a region including all of the *dcaA* gene and 194 bp of the *dcaB* gene on *D. dichloroeliminans* strain DCA1 genomic DNA. These PCR products were cloned to prepare an RD gene library of strain DCA1 by use of a pMOS Blue cloning kit (Amersham-Pharmacia) with the same protocol as described in the previous paragraph.

For enumerating copies of the *dcaA* gene in extracted environmental DNA a quantitative PCR was conducted with a GeneAmp 5700 instrument (Applied Biosystems) using the following primers: DH3F and DH3R specific for the *dcaA* gene (Table 1). The concentration of each metagenomic DNA sample was estimated using a Nanodrop instrument (Celbio, Milan, Italy), and the equivalent of 200 pg DNA was used in a 50 μl reaction mixture with DyNAmoTM HS SYBR green qPCR (Celbio, Milan, Italy) according to the instructions of the manufacturer. An initial hot start at 50°C for 2 min and denaturation at 94°C for 15 min were followed by 35 cycles consisting of denaturation at 94°C for 10 s, annealing at 55°C for 30 s, and extension at 72°C for 30 s. A final step for melting-curve analysis from 72 to 95°C, measuring fluorescence every 0.5°C, was added. Reference curves were run in every experiment by utilization of known amounts of clone RD-54 (25).

Southern blot hybridization analysis. The number of dehalogenase-encoding gene clusters in the genomic DNA of strain DCA1 was investigated in Southern hybridization experiments with two probes specific for the A and B genes of the RD cluster and labeled with digoxigenin (DIG) by random priming. Probe A was produced by PCR using primers Dca1F and DHL-REV (Table 1) for amplifying a variable region of the A gene, while primers Dca1BF and Dca1BR (Table 1) were used to amplify a probe in a conserved region of the B gene (probe B). Labeling, prehybridization, hybridization, and detection were performed with a DIG DNA labeling and detection kit (Boehringer Mannheim, Milan, Italy) according to the manufacturer's instructions (5). Genomic DNA was digested with EcoRI. Hybridization was conducted overnight at 42°C in the presence of 50% (vol/vol) formamide; two 15 min washes were performed at 50°C (low stringency) and 65°C to increase hybridization specificity.

cDNA synthesis and reverse transcription-PCR. RNA extraction was performed on 8.5 ml of 6VS culture by using a NucleoSpin RNA II kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's instructions. Total extracted RNA was treated with 10 U of DNase (Promega) at 37°C for 15 min. cDNA was synthesized using a RevertAid H Minus M-muLV reverse transcriptase kit (Promega) according to the manufacturer's instructions. Two cDNAs were synthesized with the *dcaA* sequence-specific primer DHLR2 and with the *dcaB* sequence-specific primer DcaB rev.

Nucleotide sequence accession numbers. The nucleotide sequences of the RD gene clusters were deposited in the EMBL nucleotide sequence database (GenBank/EMBL/DDBJ) under accession numbers AM183918 and AM183919 for the *D. dichloroeliminans* RD and the environmental RD, respectively.

RESULTS

Enrichment and characterization of a 1,2-DCA-dehalogenating culture. An actively 1,2-DCA-dechlorinating culture was prepared from contaminated aquifer groundwater sampled below an industrial site with a history of contamination by 1,2-DCA (25). A bacterial dehalogenating consortium was maintained through adhesion of the original culture to an active-carbon support and regular addition of high levels of 1,2-DCA and lactate. The culture, referred to here as 6VS, stably maintained the capacity of dechlorinating 1,2-DCA to ethene as the only dechlorination product, with a concomitant stoichiometric release of chloride ions (data not shown). Parallel cultures maintained without supplementation of vitamin K were much less active in 1,2-DCA dechlorination, indicating that the vitamin was important for the maintenance of the dechlorinating species in the consortium (7).

An analysis of the bacterial composition of the 6VS culture was performed by 16S rRNA gene PCR-DGGE. Several bands appeared to be enriched upon culturing in the presence of 1,2 DCA and lactate as an electron donor (data not shown). Sequencing of these bands identified 16S rRNA genes related to those of *Dehalobacter restrictus* (99% nucleotide identity over a sequence of 696 positions [U84497 in the NCBI database]) and of *D. dichloroeliminans* strain DCA1 (97% nucleotide identity over a sequence of 778 positions [AJ565938 in the NCBI database]).

Quantitative PCR specifically targeting the16S rRNA genes of the genera *Dehalobacter* and *Desulfitobacterium* was performed on the total DNA extracted from the 6VS culture. The 16S rRNA gene copy numbers of the *Dehalobacter* sp. ($2.7 \pm 1.5 \times 10^3$ molecules pg^{-1} of genomic DNA) were about eight times higher than those of the *Desulfitobacterium* sp. ($3.5 \pm 1.0 \times 10^2$ molecules pg^{-1} of genomic DNA).

PCR amplification and sequencing of a new reductive dehalogenase gene. In previous work we obtained a PCR fragment that after sequencing could be attributed to a putative novel RD (25). Starting from that sequence, by a series of inverse and direct PCRs we obtained a 7,462 bp novel RD gene cluster. Alignment of the complete gene cluster sequence, named RD-54, with known gene clusters encoding enzyme complexes active in reductive dechlorination of chlorinated aliphatics showed 98% identity (7,342 bp over 7,462 bp) with a gene cluster of *Dehalobacter restrictus* strain DSMZ 9455^T, coding for an RD active on PCE. The gene cluster included five open reading frames (ORF), named *dcaA*, *dcaB*, *dcaC*, *dcaT*, and *orf1*, preceded by a sixth ORF similar to the putative transposase associated with the gene cluster of *pceABC* and *pceT* of strain DSMZ 9455^T. All of the genes except *dcaA* were >98% identical (at the nucleotide level) to the corresponding genes of strain DSMZ 9455^T and to those of other related bacteria of the genus *Desulfitobacterium*. The ORF *dcaB* showed 98% and 97% nucleotide and amino acid identity, respectively, to *pceB* of *Desulfitobacterium* sp. strain PCE-S coding for a membrane anchor protein. The ORF *dcaC* shared 99% of both nucleotide and amino acid identity with *pceC* of strain DSMZ 9455^T, codifying a hypothetical membrane-bound regulatory protein (22). Gene *dcaT* shared 99% of both nucleotide and amino acid identity with the *pceT* of *Desulfitobacterium* sp. strain Y51, which is believed to code for a trigger factor (22, 39). The incomplete *orf1* sequence at the end of the gene cluster was 100% identical to the *orf1* gene of strain DSMZ 9455^T. The truncated transposase-like gene, directly upstream of the gene cluster of *dcaABC* and *dcaT* on the complementary strand (Fig. 1), showed 99% of both nucleotide and amino acid identity to the *tnpA* gene of strain DSMZ 9455^T (22).

The gene *dcaA* encoding the RD showed much lower identity with the corresponding *pceA* of strain DSMZ 9455^T, with 94% and 90% nucleotide and amino acid identities. A total of 59 amino acid residues over 551 differed between DcaA of RD-54 and the known RD for chlorinated ethenes (Fig. 2). A relatively high (53%) fraction of the substitutions was concentrated in two small and defined regions of the protein. The first of these two regions, between residues 123 and 179, included 19 amino acid substitutions, i.e., 32% of the total amino acid changes. The second region between residues 320 and 368 included 12 amino acid substitutions, i.e., 20% of the total amino acid changes. Other regions with a lower number of substitutions were found in the region between residues 220 and 274 and in the C-terminal region of the protein. These localized amino acid differences indicated that the *dcaA* gene might represent a new type of RD.

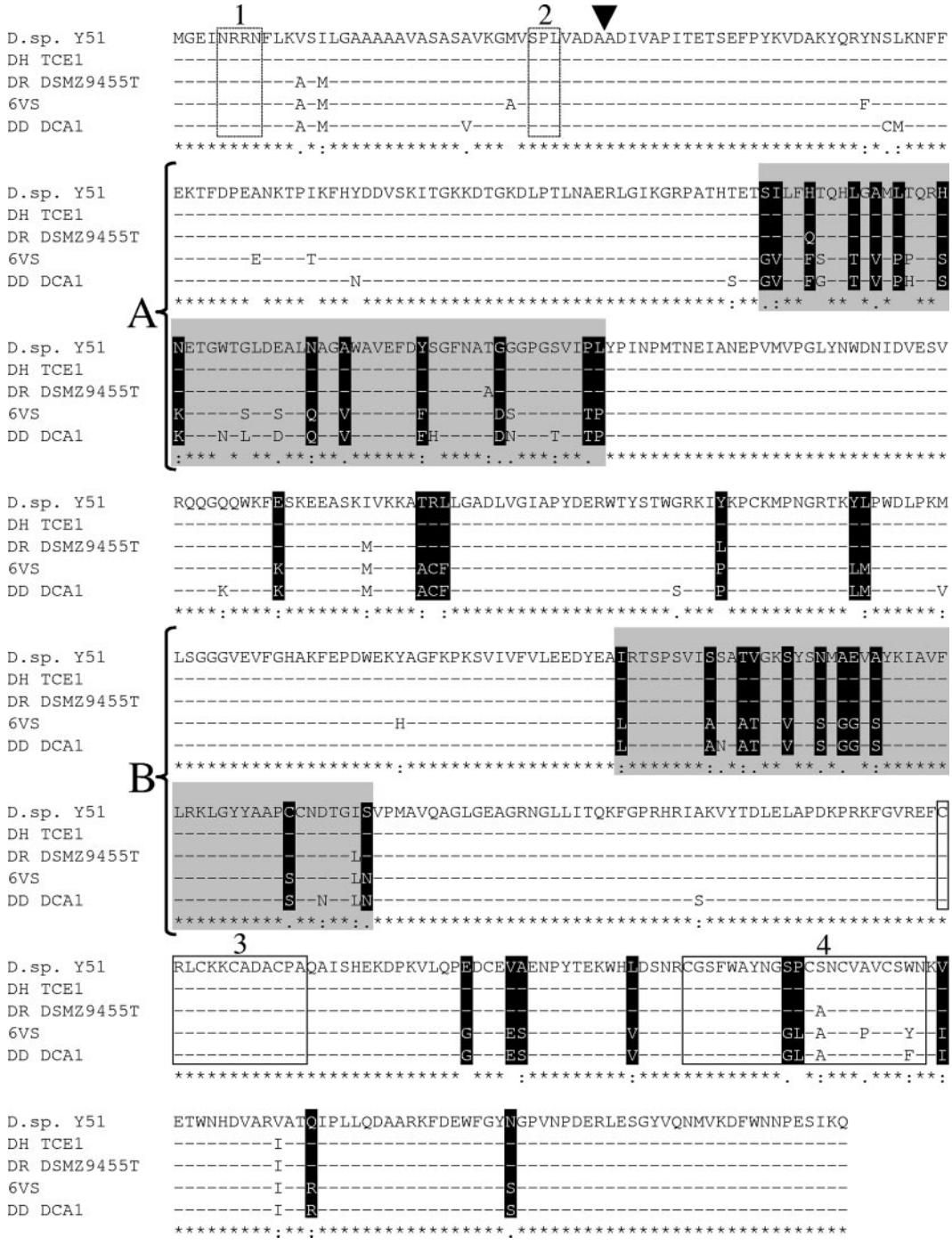

FIG. 2. Amino acid alignment of the DcaA proteins of RD-54 from culture 6VS and RD-DCA1 (DD TCA1) from *D. dichloroeliminans* strain DCA1 with PceA of *Dehalobacter restrictus* strain DSMZ 9455^T (DR DSMZ9455T) (AJ439607 in the NCBI database), *D. hafniense* strain TCE1 (DH TCE1) (AJ439608 in the NCBI database), and *Desulfitobacterium* sp. strain Y51 (D. sp. Y51) (AY706985 in the NCBI database) obtained with *tblastx* software. Rectangles 1 and 2 indicate the typical amino acid signatures of proteins translocated through a TAT system. The black triangle indicates a putative cleavage site of a signal peptide. Rectangles 3 and 4 represent the two iron-sulfur cluster binding motifs typical of RDs (21). Light gray areas A and B indicate two amino acid stretches where resides 53% of the total amino acid diversity between DcaA and PceA. The positions that differ between PceA and DcaA but are conserved between DcaA of RD-54 and DcaA of RD-DCA1 are indicated with light letters in black areas. Asterisks, colons, and dots below the alignment indicate positions identical in all the proteins, positions with a conservative substitution, and positions with a semiconservative substitution, respectively.

RD-54 copy number and transcription in response to 1,2-DCA and lactate supplementation. To understand the role of the new RD-54 gene cluster in 1,2-DCA dechlorination, *dcaA* gene copy numbers were measured by qPCR upon addition of 1,2-DCA and lactate to water samples. The copy number of *dcaA* increased from $1.98 \pm 0.2 \times 10^2$ to $2.01 \pm 0.3 \times 10^4$ copies ml^{-1} of culture after 14 days incubation.

In addition, the transcription of *dcaA* and *dcaB* was assessed

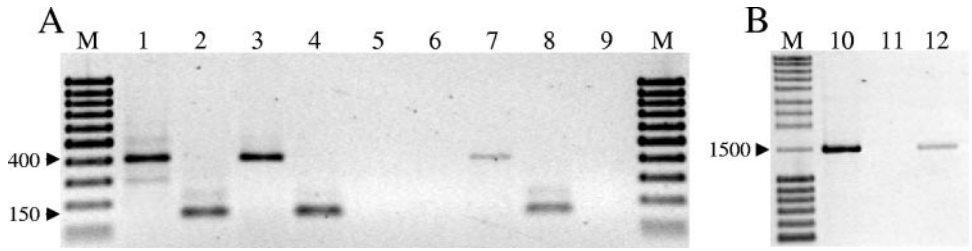

FIG. 3. Transcription of *dcaA* and *dcaB* in culture 6VS during dechlorination of 1,2-DCA. (A) A 1.2% agarose gel of the PCR experiment using specific primers for the *dcaA* gene (DHL F1, DHL F2, and DHL R1) to assess the transcription of *dcaA*. Data represent the results of experiments using PCR on genomic DNA (lanes 1 and 2), total RNA before the treatment with DNase (lanes 3 and 4), total extracted RNA following the treatment with DNase (lanes 5 and 6), and the cDNA synthesized from the pure RNA (lanes 7 and 8). Lane 9, negative control. (B) Data for primer DcaB rev (specific for *dcaB*) coupled with DHL F1. Data represent the results of experiments using PCR on genomic DNA (lane 10), total extracted RNA following the treatment with DNase (lane 11), and the cDNA synthesized from the pure RNA (lane 12). M, marker (band sizes are given in base pairs).

by RT-PCR on the total RNA extracted from the culture after 10 days of incubation in the presence of lactate and 1,2-DCA (Fig. 3). Three primers sets were used: two sets targeting *dcaA* and one for *dcaB*. RT-PCR experiments with all the three primer sets gave consistent results indicating that the two genes were transcribed in the presence of 1,2-DCA and lactate. The primer combination for the RT-PCR experiment using *dcaB* (primer DcaB rev coupled with DHL F1) resulted in the reverse transcription and amplification of the expected product, indicating that *dcaA* and *dcaB* were cotranscribed.

Identification of an RD gene cluster in *D. dichloroeliminans* strain DCA1. Specific primers were designed on the *dcaA* sequence of RD-54 to search for the RDs involved in the reductive dechlorination of 1,2-DCA in *D. dichloroeliminans* strain DCA1. Direct PCR on strain DCA1 by using primers DHL F1 and DHL R2 did not result in the amplification of any product. However, a PCR fragment of the expected size (1,944 bp) was obtained using primers PceAFor1 and DcaB rev. These primers anneal in regions conserved in all the RDs with the gene structure of *dcaABC* and *dcaT*. This PCR product was cloned, and 20 clones have been partially sequenced and showed 99.9% similarity to each other. Starting from this fragment, with a combination of four direct PCRs using primers reported in Table 1 (DCA1ups coupled with RDdca1R1, RDdca1F1 with DcaB rev, DHL-ForA with PceC-rev2, and DcaC-F2 with DCA1dcaTr), the complete sequence of the RD gene cluster (RD-DCA1) was identified in *D. dichloroeliminans* strain DCA1. To confirm the shared identity of culture 6VS and strain DCA1 dehalogenase gene clusters and to provide evidence that the newly identified gene clusters were not chimeras, long-range PCRs were performed both on the genomic DNA of strain DCA1 and on the metagenomic DNA of culture 6VS. A 4,080-long PCR product and a 3,384-bp-long PCR product were obtained from strain DCA1 and culture 6VS, respectively. Primer pair RDdca1F1/PceT-rev2 and primer pair PceAFor1/PceC-rev2 (Table 1) were used for strain DCA1 and culture 6VS, respectively. Both the fragments were completely sequenced and were shown to be identical to the gene clusters previously identified.

RD-DCA1 was preceded by a transposase that showed 99% identity (1,067 identical nucleotide positions over 1,070) to that of *D. hafniense* strain TCE1 (22) but that was different from that found in the RD-54 sequence. Alignment of the complete RD-DCA1 sequence showed 98% (4,384 positions over 4,473) and 97% (5,759 positions over 5,921) identity with the corresponding gene clusters previously identified in RD-54 gene cluster and in *D. hafniense* strain TCE1 (AJ439608), respectively. Percentages of protein and gene sequence identity and similarity between the two new RDs (RD-54 and RD-DCA1) and those involved in the dehalogenation of chlorinated ethenes are reported in Table 2. The structural genes (*dcaB*, *dcaC*, and *dcaT*) were found to be very similar and highly conserved compared to the corresponding genes of the other RDs, with nucleotide identities in the 97 to 99% range and amino acid identities and similarities in the 96 to 99% range (Table 2). The gene *dcaA* in the RD-DCA1 cluster was 98% (1,622 of 1,653 bp) identical to *dcaA* of RD-54 but only 94% identical to *pceA* genes active on chlorinated ethenes (Table 2). The putative DcaA proteins encoded by RD-54 and RD-DCA1 shared 95% identity and 98% sequence similarity. The percentages of identity to the other RDs of *Desulfitobacterium* sp. and *Dehalobacter restrictus* DSMZ 9455^T dehalogenating chlorinated ethenes decreased to 89%, with 92 to 93% sequence similarity. Figure 4 shows the phylogenetic distance between DcaA of RD-54 and RD-DCA1 and the other related genes involved in reductive dehalogenation of halogenated aliphatics. The tree indicates that the two DcaA proteins clustered in a phylogenetic branch divergent from those of the other corresponding RD proteins active on chlorinated ethenes and chlorophenol.

To search for other RD genes in strain DCA1, Southern blot hybridization experiments was performed on EcoRI-digested total genomic DNA of strain DCA1 (Fig. 5). Probe A, specific for the *dcaA* gene of strain DCA1, hybridized on two fragments of about 5,500 and 7,000 bp. Probe B, targeting both *dcaB* and *pceB* genes, hybridized on the same two fragments, indicating that *D. dichloroeliminans* strain DCA1 harbors two identical or highly similar copies of the same RD cluster.

DISCUSSION

Recently, several RDs specific for chlorinated aliphatics were characterized and sequenced, including genes from the dechlorinating-specialized genera *Dehalococcoides* (references 18 and 45 and references therein) and *Dehalospirillum* (29) and the low-G+C gram-positive genera *Clostridium* (31), *Desulfito-*

TABLE 2. Nucleotide sequence identity and predicted amino acid sequence identity and similarity between the four genes (*dcaABCT*) of RD-54 (AM183919 in the NCBI database) and the corresponding genes of RD-DCA1 of *D. dichloroeliminans* strain DCA1 (AM183918 in the NCBI database) and of *Dehalobacter restrictus* strain DSMZ 9455^T (AJ439607 in the NCBI database), *Desulfitobacterium* sp. strain Y51 (AY706985 in the NCBI database), *D. hafniense* strain TCE1 (AJ439608 in the NCBI database), and *Desulfitobacterium* sp. strain PCE-S (AY216592 in the NCBI database)[a]

Gene and microorganism	No. of positive aa[b] matches/ total no. of positions (% similarity)	No. of identical aa/ total no. of positions (% identity)	No. of identical nucleotides/ total no. of positions (% identity)
dcaA			
D. dichloroeliminans DCA1	540/551 (98)	523/551 (95)	1,622/1,656 (98)
Dehalobacter restrictus DSMZ 9455^T	515/551 (93)	496/551 (90)	1,566/1,656 (94)
D. hafniense TCE1	515/551 (93)	492/551 (89)	1,557/1,656 (94)
Desulfitobacterium sp. strain Y51	515/551 (93)	491/551 (89)	1,557/1,656 (94)
Desulfitobacterium sp. strain PCE-S	512/551 (92)	491/551 (89)	1,556/1,656 (94)
dcaB			
D. dichloroeliminans DCA1	104/105 (99)	103/105 (98)	314/318 (99)
Dehalobacter restrictus DSMZ 9455^T	102/105 (97)	101/105 (96)	313/318 (98)
D. hafniense TCE1	102/105 (97)	102/105 (97)	314/318 (98)
Desulfitobacterium sp. strain Y51	102/105 (97)	102/105 (97)	313/318 (98)
Desulfitobacterium sp. strain PCE-S	102/105 (97)	102/105 (97)	314/318 (98)
dcaC			
D. dichloroeliminans DCA1	352/367 (96)	349/367 (95)	1,070/1,104 (97)
Dehalobacter restrictus DSMZ 9455^T	366/367 (99)	365/367 (99)	1,101/1,104 (99)
D. hafniense TCE1	365/367 (99)	362/367 (98)	1,096/1,104 (99)
Desulfitobacterium sp. strain Y51	362/367 (98)	362/367 (98)	1,096/1,104 (99)
dcaT			
D. dichloroeliminans DCA1	313/316 (99)	312/316 (98)	945/951 (99)
Dehalobacter restrictus DSMZ 9455^T	315/316 (99)	312/316 (98)	946/951 (99)
D. hafniense TCE1	312/316 (98)	308/316 (97)	938/951 (98)
Desulfitobacterium sp. strain Y51	315/316 (99)	313/316 (99)	945/951 (99)

[a] For strain PCE-S only the sequences of *dcaA* and *dcaB* were available.
[b] aa, amino acids.

bacterium (23, 41), and *Dehalobacter* (23). All the RDs characterized to date show activity towards chlorinated ethenes, but only a few can dehalogenate 1,2-DCA and other alkanes (45). Phylogenetic analyses showed that RDs for chlorinated aliphatics are divided into several groups with a low level of similarity. Most of the sequences are located in two main groups: the first with RDs from *Dehalococcoides* sp. and a second with RDs from *Desulfitobacterium* sp. and *Dehalobacter* sp. (13, 18, 23, 45). Genetic and biochemical characterization of RDs has not yet identified which amino acid motifs might be involved in the substrate specificity. Based on simultaneous transcription of different RDs in the presence of the same chlorinated compound, some authors proposed that substrate range and specificity can be governed by different corrinoid cofactors interacting with holoenzymes with few sequence differences (45).

No RD selective for halogenated alkanes has yet been described. Recently, *D. dichloroeliminans* strain DCA1 was shown to dechlorinate 1,2-DCA and other vicinal dichlorinated alkanes, but not chlorinated ethenes, by a dichloroelimination reaction (6). In a previous study we showed that addition of lactate to groundwater contaminated exclusively by 1,2-DCA had led to the enrichment of *Desulfitobacterium* sp. (25). The enriched culture 6VS (originating from that groundwater) was dominated by *Dehalobacter* and *Desulfitobacterium* spp., as shown by 16S rRNA PCR-DGGE and sequencing results. These data were also confirmed by clone prevalences in 16S rRNA gene libraries, showing the enrichment of these two genera following addition of an electron donor to the groundwater (data not shown). Quantitative real-time PCR experiments showed a *Dehalobacter restrictus* 16S rRNA gene concentration seven times higher than that of *Desulfitobacterium* sp. All these data indicated that these genera play an important role in 1,2-DCA reductive dechlorination in the 6VS culture. Besides, the specialization of *D. dichloroeliminans* strain DCA1 in dechlorinating alkanes should have selected RDs specifically adapted to 1,2-DCA and chlorinated alkanes in the microbe's genome. These RDs should display signature structural motifs linked to the enzyme specificity for 1,2-DCA (45).

By using a PCR assay recently developed for the amplification of unknown dehalogenases (25, 33) and the following inverse and direct PCRs, we were able to sequence very similar RD gene clusters from the 6VS culture and strain DCA1.

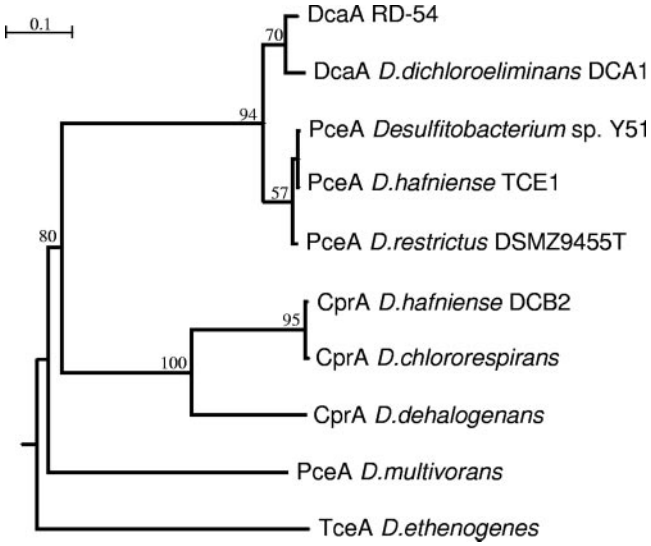

FIG. 4. Neighbor-joining tree constructed using ClustalW software (http://clustalw.genome.jp/) and showing the phylogenetic relationship between DcaA of the 6VS culture (DcaA RD-54) and of *D. dichloroeliminans* strain DCA1 with other A subunits of genetically characterized RDases: PceA of *Dehalobacter restrictus* strain DSMZ 9455^T (AJ439607 in the NCBI database), *Desulfitobacterium hafniense* strain TCE1 (AJ439608 in the NCBI database), and *Desulfitobacterium* strain Y51 (AY706985 in the NCBI database). Other data represent the results of investigations of the chlorophenol RDases (CprA) of *Desulfitobacterium chlororespirans* (AF204275 in the NCBI database), *Desulfitobacterium hafniense* strain DCB-2 (AY013365 in the NCBI database), and *Desulfitobacterium dehalogenans* (AF115542 in the NCBI database) and of TceA of *Dehalococcoides ethenogenes* (AF228507 in the NCBI database) and PceA of *Dehalospirillum multivorans* (AF022812 in the NCBI database). The numbers at each branch point represent bootstrap percentages calculated from 1,000 replicate trees. The scale bar represents the sequence divergence.

These RD clusters included a sequence of four genes (*dcaABC* and *dcaT*) similar to the RD clusters of *Desulfitobacterium* and *Dehalobacter* spp. The only significantly divergent sequence was that of *dcaA*, which showed only 92 to 93% nucleotide similarity with *pceA* of RD clusters active on chlorinated ethenes, while all the other genes had 97 to 99% similarity.

DcaA amino acid sequences from culture 6VS and strain DCA1 showed distinctive characteristics common to other RDs, including the twin-arginine motif (RRxFLK) found in proteins which are translocated in the periplasm by a TAT system (3, 4) and contain redox cofactors (Fig. 2) (23), a central stretch of the signal peptide rich of hydrophobic amino acids (especially alanine), and the same ADA ↓ ADIVA motif present at the cleavage site of the signal peptide of the RD of *Dehalobacter restrictus* DSMZ 9455^T (23). Two iron-sulfur cluster binding motifs (Fig. 2) typical of almost all the RDs characterized so far were found in the DcaA C-terminal region of both RD-54 and RD-DCA1. These iron-sulfur clusters are probably involved in electron transfer to the active site containing the corrinoid factor (13, 29, 44). The first iron-sulfur cluster had a sequence identical to that of the corresponding motif in PceA of *Dehalobacter restrictus* DSMZ 9455^T (Fig. 2), showing the consensus sequence ($CX_2CX_2CX_3CP$) with cysteine in fixed positions typical of RD. The second iron-sulfur cluster showed some differences with respect to strain DSMZ 9455^T, while the consensus motif $CX_{10}CX_2CX_3C$ was preserved (Fig. 2). This second iron-sulfur cluster-binding motif is known to be less conserved among RDs (23).

Between 10 and 11% of the amino acid residues of RD-54 and RD-DCA1 DcaA differed from those of the PceA proteins. About 53% of this diversity (31 and 36 amino acids for DcaA of RD-54 and RD-DCA1, respectively) was localized in two regions (regions A and B in Fig. 2) that represented only 19% (104 amino acids over 551) of the total DcaA residues. The function of these two regions is presently unknown, but (i) the sequence conservation despite the different geographic origins of the cultures (Italy for culture 6VS and Belgium for

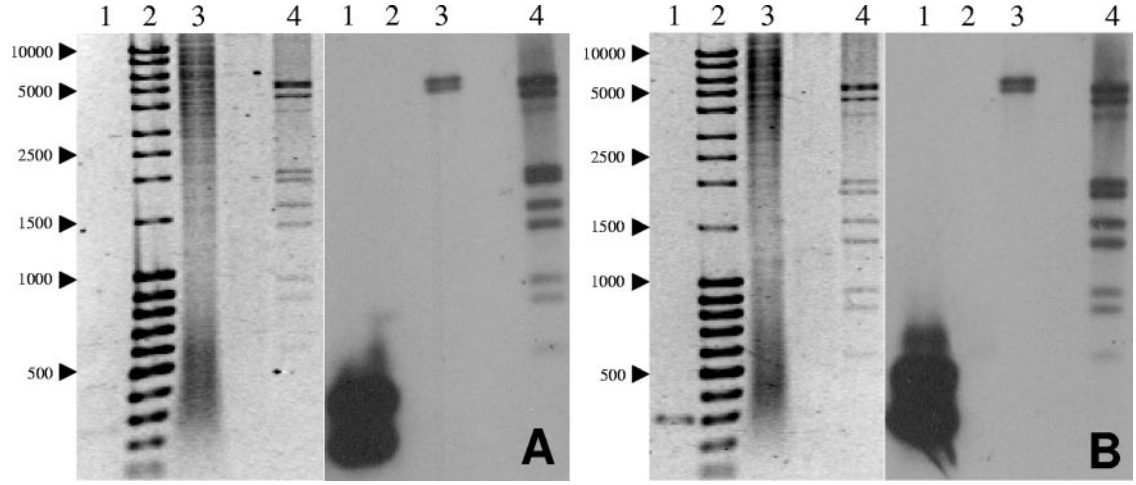

FIG. 5. (A) Results of 1.2% agarose gel and relative Southern blot analysis using probe B, universal for the B genes known to date (*dcaB*, *pceB*). Lanes: 1, positive control for probe B; 2, marker mix (Fermentas); 3, EcoRI restriction digestion of total genomic DNA of strain DCA1; 4, DIG-labeled ladder. (B) Results of 1.2% agarose gel and relative Southern blot analysis using probe A, specific for *dcaA* gene of strain DCA1. Lanes: 1, positive control for probe A; 2, marker mix (Fermentas); 3, EcoRI restriction digestion of total genomic DNA of strain DCA1; 4, DIG-labeled ladder. Dimensions of the main marker bands in base pairs are shown on the left of each panel.

D. dichloroeliminans strain DCA1), (ii) the persistence and enrichment of the *dcaA* gene in dehalogenating cultures as measured by quantitative real-time PCR, (iii) the transcription of *dcaA* and *dcaB* genes in the 6VS culture in the presence of 1,2-DCA and lactate, and (iv) the substrate specificity for 1,2-DCA and other vicinal dichloroalkanes exhibited by *D. dichloroeliminans* DCA1 (6) suggest the possibility that these two regions could be involved in 1,2-DCA recognition and in the substrate specificity of RDs in general. Considering the RD redundancy found in the genomes of other dechlorinators, such as *Dehalococcoides* sp. (13, 18, 37, 45), RD gene library and Southern blot hybridization analysis were performed to survey the RD genes in the genome of strain DCA1. Here we found two gene sequence copies identical or highly similar to that of *dcaA*. These results confirm that RD-DCA1 is the most probable candidate dehalogenase for dehalogenation of 1,2-DCA in strain DCA1 and that the two enzyme regions differing from PceA dehalogenase are signature stretches correlated to 1,2-DCA dechlorination.

From an evolutionary point of view, the finding of only one (*dcaA*) of four genes in the RD clusters significantly diverging from the corresponding genes of the RD clusters implicated in chloroethene dechlorination (Table 2) can be explained by an accumulation of mutations or by recombination with other RDs. The localization of divergent sequence stretches in only certain regions of DcaA (Fig. 2) suggests that recombination rather than mutation is the process that occurred in the *dcaA* genes from culture 6VS and from strain DCA1. A putative RD chimera was recently described in investigations of the genome of *Dehalococcoides* sp. strain CBDB1, in which the *rdhA* gene *cbdbA1508* appeared to be the result of recombination between a unique *rdhA* gene N-terminal sequence and the C-terminal sequence of another (*cbdbA1588*) of the 32 RDs in the genome of strain CBDB1 (18).

By comparing the RD pools in the genomes of *Dehalococcoides* sp. strains 195 and CBDB1, Kube et al. concluded that the genome regions containing RDs have high plasticity and that RD genes are under intense evolutionary pressure (18). The findings of Kube et al. (18) raised the intriguing possibility that *Dehalococcoides* sp. could have acquired its dechlorinating abilities recently, possibly in an adaptive response to anthropogenic release of chlorinated solvents in the environment (38). To estimate the evolution rate of the new *dcaA* genes of RD-54 and RD-DCA1 with respect to the most similar *pceA* of *Dehalobacter restrictus* DSMZ 9455^T, we calculated the ratio of dN to dS nucleotide changes in the amino acid sequences according to the method of Nei and Gojobori (28). The dN/dS ratios of $dcaA_{RD\text{-}54}$ and $dcaA_{RD\text{-}DCA1}$ were 1.28 ± 0.018 ($dN = 0.058 \pm 0.008$; $dS = 0.045 \pm 0.010$) and 1.51 ± 0.017 ($dN = 0.062 \pm 0.008$; $dS = 0.041 \pm 0.009$), respectively. Values larger than 1 are indicative of a fast evolution due to positive selection (9). Indeed, both the groundwater from which the 6VS culture was obtained (25) and the water-saturated soil from which *D. dichloroeliminans* strain DCA1 was isolated (6) had experienced contamination with 1,2-DCA for more than 30 years.

Another feature indicating the plasticity of the RD gene clusters is their physical association with mobile genetic elements that can play a key role in the evolutionary process for the development of new catabolic pathways (34, 42). RDs previously identified in the genera *Desulfitobacterium* and *Dehalobacter* were found to be associated with transposase-like sequences (22). In particular, Maillard et al. (22) identified in *D. hafniense* strain TCE1 the catabolic transposon *Tn*-Dha1 containing the *pceABC* and *pceT* genes responsible for PCE reductive dechlorination and probably mediating transposition activity. *Dehalobacter restrictus* and *D. hafniense* strain TCE1 *pceABC* and *pceT* gene clusters have 100% identity, but the transposase upstream of the RD gene cluster of *Dehalobacter restrictus* is on the complementary strand of the *pceA* gene, is truncated, and is very different from that of strain TCE1 (22). The RD-54 gene cluster of culture 6VS encodes a transposase-like sequence almost identical to that of *Dehalobacter restrictus*, while the region upstream the RD-DCA1 gene cluster of *D. dichloroeliminans* strain DCA1 is highly similar to that of the transposase of the *Tn*-Dha1 transposon of *D. hafniense* strain TCE1. The presence of complete or truncated and very similar transposase genes is not sufficient to allow speculation on the presence of active transposable elements but could be associated with a past event of transfer or rearrangements that further confirm their plasticity. The transposase sequence identity and the qPCR results suggest that the *Dehalobacter* population in the mixed culture could be the carrier of the RD-54 gene cluster.

In summary, two independent cultures, those of a 6VS enrichment and of *D. dichloroeliminans* strain DCA1, having different origins but isolated from sites exposed to similar selective pressures, were enriched on 1,2-DCA with respect to electron acceptors and the resulting enrichments were found to have similar RD sequences, suggesting that these could be linked to the dehalogenation of 1,2-DCA. Although the functions of the RDs should be further characterized, the new sequences are useful for the development of molecular tools to predict and monitor dechlorination process in sites contaminated by 1,2-DCA.

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