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Research Article

Diverse Reductive Dehalogenases Are Associated with Clostridiales-Enriched Microcosms Dechlorinating 1,2-Dichloroethane

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The achievement of successful biostimulation of active microbiomes for the cleanup of a polluted site is strictly dependent on the knowledge of the key microorganisms equipped with the relevant catabolic genes responsible for the degradation process. In this work, we present the characterization of the bacterial community developed in anaerobic microcosms after biostimulation with the electron donor lactate of groundwater polluted with 1,2-dichloroethane (1,2-DCA). Through a multilevel analysis, we have assessed (i) the structural analysis of the bacterial community; (ii) the identification of putative dehalorespiring bacteria; (iii) the characterization of functional genes encoding for putative 1,2-DCA reductive dehalogenases (RDs). Following the biostimulation treatment, the structure of the bacterial community underwent a notable change of the main phylotypes, with the enrichment of representatives of the order *Clostridiales*. Through PCR targeting conserved regions within known RD genes, four novel variants of RDs previously associated with the reductive dechlorination of 1,2-DCA were identified in the metagenome of the *Clostridiales*-dominated bacterial community.

1. Introduction

Chlorinated compounds are among the major global environmental contaminants [1]. A large number of compounds of this class of chemicals have been produced in big quantities for several applications in industry and agriculture such as biocides, flame retardants, solvents, and intermediates for the production of polymers (e.g., PVC) [1, 2]. Their widespread diffusion and use resulted in the massive release in the environment, with consequent concerns for human health due to the persistence, tendency to bioaccumulate, and proven toxicity [2, 3]. Due to the physicochemical properties,

most halogenated compounds are recalcitrant to aerobic dehalogenation and tend to accumulate in anoxic ecosystems (e.g., soils and groundwater aquifers). For this reason, many of the research efforts of the last decades, aimed at defining efficient remediation approaches, were focused on the investigation of anaerobic degrading potential of microbial cultures enriched/isolated from typical anoxic environments. Chlorinated solvents in these conditions can undergo biologically mediated degradation through either oxidative, fermentative, or reductive processes [4]. Particular interest has been focused on the third kind of biodegradation process, since several studies have highlighted the high dechlorinating

performances of pure and mixed microbial cultures through reductive dehalogenation [5–10]. The peculiarity of this process is that the chlorinated molecule is the terminal electron acceptor of the membrane-bound electron transport chain coupled to the generation of energy in the form of ATP [4].

Among the wide variety of chlorinated solvents, 1,2-dichloroethane (1,2-DCA) is considered one of the major pollutants, being one of the most widespread contaminating groundwater worldwide and being classified as a possible human carcinogenic agent by many environmental agencies [2]. 1,2-DCA can undergo either partial or complete detoxification in anoxic conditions through three different mechanisms: dichloroelimination, reductive hydrogenolysis, and dehydrochlorination [5]. Among these, only the first mechanism leads to the production of the harmless end-product ethylene, while the other two generate molecules whose toxicity is even higher than 1,2-DCA, in particular the carcinogenic vinyl chloride (VC). Key enzymes involved in this anaerobic dehalogenating metabolism are the reductive dehalogenases (RDs), a class of cobalamin-dependent oxygen-sensitive enzymes, usually associated with the membranes and capable of replacing halogen atoms with hydrogen ones from the carbon backbone of the molecules [4, 11]. Different studies have unveiled details about structure and function of some enzymes belonging to this class [12–14]. Only recently, novel RDs sequences were correlated with 1,2-DCA dechlorination to ethene in a 1,2-DCA dehalogenating enrichment culture containing a *Dehalobacter* sp. WL (rdhA1, rdhA2, and rdhA3) [15] and *in situ* in the upper water layer of a double layer aquifer contaminated by 1,2-DCA (RD54) [16]. The enrichment culture setup from the upper layer of the aquifer (culture 6VS) contained both *Dehalobacter* and *Desulfitobacterium* spp. In addition to the two just cited representatives of the phylum Firmicutes, only few other bacterial strains have been identified so far as capable of detoxifying 1,2-DCA to ethylene via dichloroelimination. Papers [17, 18] were the first to report the ability of two *Chloroflexi* representatives, respectively, *Dehalococcoides ethenogenes* strain 195 and *Dehalococcoides* sp. strain BAV1 to grow on 1,2-DCA as electron acceptor producing ethylene as the main end product. A peculiarity of the species of this genus is their capability to grow exclusively on chlorinated compounds as electron acceptor. Other representatives of the phylum Chloroflexi with the ability to grow on 1,2-DCA described recently are two strains of the genus *Dehalogenimonas*: *D. lykanthroporepellens* [19] and *D. alkenigignens* [20], both characterized by the ability to degrade high concentration of 1,2-DCA up to 8.7 mM [21].

In the present work, the dechlorinating bacterial microbiome in the lower layer of the same aquifer investigated by [16] has been characterized in terms of structure and functionality, before and after the supplement with lactate. We have investigated (i) the response of the indigenous microbial community to lactate treatment, (ii) the key microbial dehalogenating bacteria, and (iii) the RDs involved in the dehalogenation process.

2. Materials and Methods

2.1. Preparation of Enrichment Cultures. Evaluation of biodegradation of 1,2-DCA was carried out in anaerobic microcosms set-up with groundwater collected from the lower layer (from 14 m to 40 m deep) of an aquifer previously studied in northern Italy [7, 9, 16], heavily polluted exclusively by 1,2-DCA more than 30 years ago. Concentration of the contaminant in the lower aquifer was about $197 \pm 23 \text{ mg L}^{-1}$ and it was maintained the same during preparation of anaerobic cultures. The other chlorinated ethane and ethene were not detected. Thirty mL triplicate microcosms were assembled in 50 mL vials under an atmosphere of 80% N_2 , 15% CO_2 , and 5% H_2 in the anaerobic glove-box Simplicity 888 (Plas-Labs, USA). Culturing medium consisted of a 1:200 dilution of a trace elements solution (12.8 g L^{-1} nitrilotriacetic acid, 1.35 g L^{-1} $\text{FeCl}_3 \cdot 6 \text{ H}_2\text{O}$, 0.1 g L^{-1} $\text{MnCl}_2 \cdot 4 \text{ H}_2\text{O}$, 0.024 g L^{-1} $\text{CoCl}_2 \cdot 6 \text{ H}_2\text{O}$, 0.1 g L^{-1} $\text{CaCl}_2 \cdot 2 \text{ H}_2\text{O}$, 0.1 g L^{-1} ZnCl_2 , 0.025 g L^{-1} $\text{CuCl}_2 \cdot 2 \text{ H}_2\text{O}$, 0.01 g L^{-1} H_3BO_3 , 0.024 g L^{-1} $\text{Na}_2\text{MoO}_4 \cdot 2 \text{ H}_2\text{O}$, 1 g L^{-1} NaCl , 0.12 g L^{-1} $\text{NiCl}_2 \cdot 6 \text{ H}_2\text{O}$, and 0.026 g L^{-1} $\text{Na}_2\text{SeO}_3 \cdot 5 \text{ H}_2\text{O}$), a supplementary salt solution (43 mg L^{-1} NH_4Cl , 0.5 g L^{-1} KH_2PO_4 , 0.2 g L^{-1} $\text{MgCl}_2 \cdot 6 \text{ H}_2\text{O}$, and 0.01 g L^{-1} $\text{CaCl}_2 \cdot 2 \text{ H}_2\text{O}$), 0.05% (w/v) yeast extract, 0.5 mM 4-(2-hydroxyethyl) piperazine-1-ethanesulfonic acid/NaOH (Hepes/NaOH) solution pH 7.0, cysteine 1 mM, and vitamin B_{12} 50 mg L^{-1} . Lactate at final concentration of 5 mM was used as the only carbon source and electron donor [22]. Control microcosms were prepared by incubating parallel vials containing the same culturing medium with filter-sterilized groundwater samples. All microcosms were sealed with teflon-faced septa and aluminum crimp seals and statically incubated in the dark at 23°C .

Concentration of 1,2-DCA and of its possible degradation products, ethane and VC, was evaluated by the injection of $500 \mu\text{L}$ samples of headspace of the microcosms in a Gas Chromatograph/Flame Ionization Detector (GC/FID) Agilent 7694 equipped with a DB624 column (J&W Scientific, Folsom, CA). The temperature of the oven and of the detector was set at 80 and 200°C , respectively. 1,2-DCA limit of detection was $1.0 \mu\text{g L}^{-1}$.

2.2. Genomic DNA Isolation. Groundwater and microcosm samples, respectively, 30 and 1.5 mL (samples withdrawn from replicate cultures were pooled together for a total final volume of 4.5 mL), were filtered using Sterivex filters (Millipore, Milan, Italy). Total genomic DNA was extracted from the filtered bacterial cells by incubating the filter with 2 mL of a lysis solution containing 1 mg mL^{-1} lysozyme, 1% (w/v) sodium dodecyl sulphate, and 0.5 mg mL^{-1} proteinase K and purified as previously described by Murray et al. [23].

2.3. PCR Amplification of Bacterial and Archaeal 16S rRNA and RD Genes. Bacterial 16S rRNA gene was amplified from the groundwater metagenome using universal primers 27f and 1492r [24] with the following reaction concentrations in a final volume of $50 \mu\text{L}$: 1X PCR buffer, 1.5 mM MgCl_2 , 0.12 mM

dNTPs, 0.3 μM of each primer, and 1 U of Taq polymerase. Thermal protocol used was the following: initial denaturation at 94°C for 5 minutes, followed by 5 cycles consisting of denaturation at 94°C for 1 minute, annealing at 50°C for 1 minute, and extension at 72°C for 2 minutes and subsequently by 30 cycles consisting of denaturation at 94°C for 1 minute, annealing at 55°C for 1 minute, and extension at 72°C for 2 minutes. A final extension at 72°C for 10 minutes was performed.

PCR with specific primers for Archaea was attempted in order to investigate the 16S *rRNA* diversity of this group of prokaryotes. A first step was carried out using universal archaeal forward primers 21f and 1492r, using the same reaction mix and thermal protocol presented elsewhere [25]. Since the first PCR step did not give any amplicon, a second round of PCR using primers PARCH 340F and 934R was attempted, as previously described by Cytryn et al. [26]. However, also this second amplification attempt did not result in any PCR product.

A 2000 bp region of the reductive dehalogenase gene cluster previously identified by Marzorati and colleagues [16] was amplified using primers PceAFor1 (5'-ACGT GCA ATT ATT ATT AAG G-3') and DcaBRev (5'-TGG TAT TCA CGC TCC GA-3'), in order to construct a gene library of the functional genes encoding for the RD specific for 1,2-DCA degradation. The reaction mix was prepared as follows: 1X PCR buffer, 1.5 mM MgCl₂, 0.2 mM dNTPs, 0.6 μM of each primer, and 1 U of Taq polymerase in a final volume of 25 μL . The thermal consisted of an initial denaturation at 94°C for 3 minutes, followed by 31 cycles of denaturation at 94°C for 30 seconds, annealing at 54°C for 1 minute, extension at 72°C for 2 minutes, and subsequently a final extension at 72°C for 7 minutes.

2.4. 16S *rRNA* and RD Genes Libraries. Cloning reactions were performed with pGEM cloning kit (pGEM-T Easy Vector Systems, Promega, Milan, Italy) following the instructions of the manufacturer. Sixty ng of PCR product was used for each cloning reaction, maintaining a molar ratio insert : vector of 3 : 1. A PCR assay was performed on white positive colonies to amplify the insert using primers T7 (3'-CTA ATA CGA CTC ACT ATA GGG-5') and SP6 (3'-ATT TAG GTG ACA CTA TAG AAT A-5'). PCR products were purified with QIAquick PCR Purification Kit (Qiagen, Milan, Italy) according to the manufacturer's instructions.

2.5. 16S *rRNA* Gene Phylogenetic and RDs Diversity Analyses. Clones from bacterial 16S *rRNA* and RD genes libraries were sequenced, respectively, with primers 27F and PceAFor1, using the ABI Prism BigDye terminator cycle sequencing kit (Applied Biosystems, Milan, Italy) and an ABI 310 automated sequencer (Applied Biosystems). Sequences were edited with software Chromas Lite version 2.01. Sequences of the 16S *rRNA* bacterial libraries were checked for chimeric PCR products using DECIPHER online software tool [27] and nonchimeric sequences were then used to define operational taxonomic units (OTUs) at 99% of similarity (OTU99) using DOTUR [28]. Shannon diversity index (H') was calculated

using software PAST version 3.02 [29]. The sequences of the OTU representatives were analysed using the Basic Local Alignment Search Tool (BLAST) of the online GenBank database [30] and by the CLASSIFIER Match Tool version 2.6 of Ribosomal Database Project II (RDP II) [31]. Pareto-Lorenz distribution curves (PL curves) [32, 33] were constructed based on the 16S *rRNA* gene clone library results, in order to graphically evaluate the community organization (Co) of the bacterial consortia as described elsewhere [34].

Identification of the closest relative match for the RDs libraries was carried out comparing the sequences with BLAST. Sequences of functional gene libraries were used to construct neighbour-joining phylogenetic tree, with bootstrap of 1000 repetitions, and compute the evolutionary distances through Kimura's two-parameter model using software MEGA version 5 [35]. Alignment of amino acids sequences of the functional genes deduced from the nucleotide sequences of the RDs libraries was carried as described elsewhere [36] in order to identify characteristic amino acid residues conserved in all RDs.

2.6. Nucleotide Sequence Accession Numbers. Nucleotide sequences of all clones identified in this study were deposited in the EMBL nucleotide sequence database (GenBank/EMBL/DDBJ) under the accession numbers FM210335, FM204948 to FM204979 for bacterial 16S *rRNA* genes, and FM204931 to FM204934 for RDs sequences.

3. Results and Discussion

3.1. Structure and Diversity of the Bacterial Community before and after the Biostimulation. A triplicate series of anaerobic microcosms with a concentration of 1,2-DCA of $197 \pm 23 \text{ mg L}^{-1}$ was set up using groundwater from the lower layer of a double aquifer contaminated by 1,2-DCA analogously to the experiments previously run for the upper layer of the same aquifer system [9]. Following the addition of 5 mM lactate, all the microcosms readily degraded 1,2-DCA in 15 days, with an average dechlorination rate of $13.1 \pm 1.9 \text{ mg L}^{-1} \text{ day}^{-1}$. Ethane accumulated as the only end product while the toxic intermediate VC was always below the detection limit, suggesting that degradation of 1,2-DCA occurred only via dichloroelimination [22]. The analogous biostimulation treatment with groundwater from the upper layer [9] gave considerably higher degradation rate of $69.4 \pm 2.2 \text{ mg L}^{-1} \text{ day}^{-1}$. It can be speculated that this almost-four times statistically significant difference (as determined by Student's *t*-test with $P < 0.000001$) between the two layers was possibly due to differences in the enriched dechlorinating species.

The bacterial diversity of the community before (t_0) and after (t_1) the biostimulation treatment was evaluated by establishing 16S *rRNA* gene clone libraries. Differently from what was observed previously on the upper layer of the aquifer [9], PCR with specific primers for Archaea did not result in any amplicon either before or after lactate amendment, even after a second round of PCR using nested

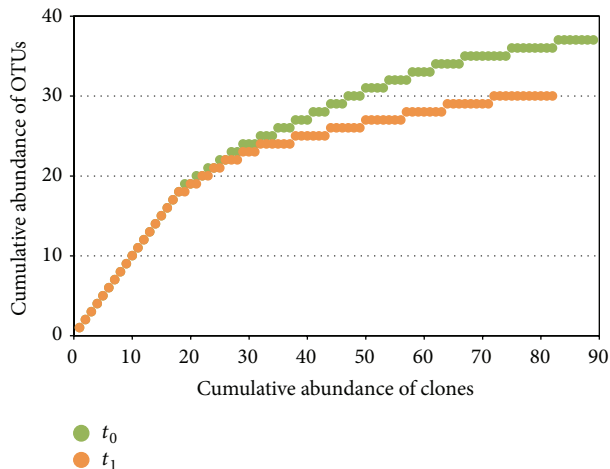


FIGURE 1: Rarefaction curves calculated for the bacterial *16S rRNA* gene clone libraries, before (t_0) and after (t_1) the biostimulation treatment with lactate.

primers. This suggests that in the lower aquifer Archaea are not implicated in the dechlorination process.

The bacterial libraries were made of 91 clones each. Chimera check allowed excluding 6.0% of all the sequences obtained, lowering the number of clones to 89 and 82 for t_0 and t_1 , respectively. Good coverage of the dominant OTUs was confirmed with rarefaction analysis of the clone libraries (Figure 1). The diversity of the bacterial communities was evaluated by means of two parameters: (i) Shannon index (H'), which allowed describing the species richness, and (ii) evenness index, used to describe the relative abundance among species within the communities. Shannon index, which accounts for both abundance and evenness of the species present, was 3.33 in the lower aquifer with respect to 1.91 in the upper one, indicating that the lower aquifer hosted greater species diversity than the upper one before the treatment. At t_1 after lactate amendment the Shannon index in the lower aquifer decreased (2.88 versus 3.33), while in the upper aquifer it remained almost unchanged (1.81 versus 1.91). The small H' variation in the lower aquifer suggests that relatively limited changes in the biodiversity of the bacterial community occurred after the biostimulation treatment.

The PL curves, used as a graphical estimator of the Co [32, 33], confirmed the little bacterial diversity change in the lower aquifer, in response to the biostimulation treatment (Figure 2). Co curves at t_0 showed a situation where 20% of the OTUs represented about 48% of the total abundance of clones. After the lactate treatment, this proportion grew to 58%, indicating that both communities were characterized by a relatively moderate organization. It can be speculated that the bacterial community of the lower aquifer was characterized by a slight dominance both before and after the biostimulation treatment and sudden changes in the environmental conditions, as those determined by the supplement of lactate, would change the dominant species but would not influence the overall Co and evenness structure of the community.

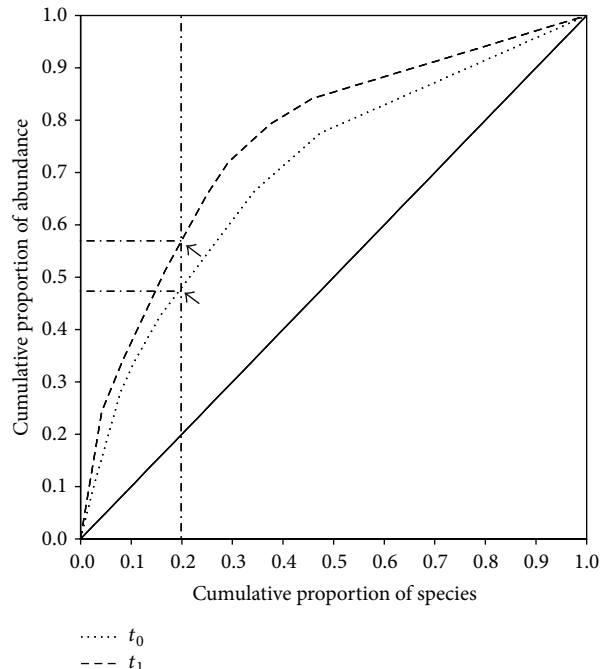


FIGURE 2: Pareto-Lorenz distribution curves representation of the community organization (Co) of the microbial communities before (t_0 , dotted line) and after (t_1 , dashed line) the treatment with lactate. The continuous line represents the perfect evenness. Black arrows indicate the OTU cumulative proportion of abundances corresponding to an OTU cumulative proportion of 20%.

The 171 clones obtained in the two libraries were grouped in 60 distinct OTUs. A summary of the representatives of each OTU identified through BLAST and CLASSIFIER is presented in Table 1, together with the number of clones of each OTU occurring before and after the biostimulation treatment. Thirty-eight of the 60 OTUs were detected before the lactate amendment and 24 after it, with only two OTUs detected both at t_0 and at t_1 , respectively, affiliated to uncultured Clostridiales and to *Sulfuricurvum* sp. The bacterial community at t_0 was characterized by a wider diversity, with dominating sequences belonging to Proteobacteria phylum (Table 1, Figure 3): in order of abundance δ - (38 clones describing 15 OTUs), β - (26 clones describing 11 OTUs), and ϵ -Proteobacteria (15 clones describing 4 OTUs). Within the δ -Proteobacteria, all the sequences were closely related to genus *Geobacter* (97–100% identity), the majority of which were affiliated to uncultured *Geobacter* sp. and *Geobacter thiogenes* (15 clones each). Species of the genus *Geobacter* were commonly found in freshwater sediments and subsurface environments [37]. Previously, de Wever and colleagues [38] described the ability of *Geobacter thiogenes* to dechlorinate trichloroacetic acid. Another representative of the *Geobacter* clade, *G. lovleyi* (6 clones), a known tetrachloroethene-dechlorinating bacterium [39], was also identified. Within the β - and ϵ -Proteobacteria groups, the most represented phylotypes were closely related to *Hydrogenophaga taeniospiralis* (11 clones) and *Sulfuricurvum kujiense* (10 clones). These two genera are environmental

TABLE 1: Summary of the OTU representatives, identified in the 16S rRNA gene libraries.

| OTU | Clones | | Basic Local Alignment Search Tool-GenBank | | CLASSIFIER Match Tool-Ribosomal Database Project II | | | |
|-----|--------|-------|---|-----------|---|-----------------------|----------------------------------|--------------------------|
| | t_0 | t_1 | Closest described relative | Acc. n° | % identity | Phylogenetic group | Closest classified relative | % certainty ^A |
| 1 | 3 | 0 | <i>Geobacter thiogenes</i> | NR_028775 | 98.96 | Deltaproteobacteria | <i>Geobacter</i> | 100 |
| 2 | 3 | 0 | <i>Geobacter thiogenes</i> | NR_028775 | 98.78 | Deltaproteobacteria | <i>Geobacter</i> | 100 |
| 3 | 8 | 0 | <i>Geobacter thiogenes</i> | NR_028775 | 99.09 | Deltaproteobacteria | <i>Geobacter</i> | 100 |
| 4 | 3 | 0 | Unc. bacterium | AM410013 | 97.34 | Deltaproteobacteria | <i>Geobacter</i> | 100 |
| 5 | 1 | 0 | Unc. <i>Geobacter</i> sp. | FM204959 | 98.63 | Deltaproteobacteria | <i>Geobacter</i> | 100 |
| 6 | 1 | 0 | Unc. <i>Geobacter</i> sp. | EU266833 | 98.62 | Deltaproteobacteria | <i>Geobacter</i> | 100 |
| 7 | 3 | 0 | Unc. <i>Geobacter</i> sp. | AY752765 | 98.56 | Deltaproteobacteria | <i>Geobacter</i> | 100 |
| 8 | 1 | 0 | Unc. <i>Geobacter</i> sp. | AY752765 | 98.42 | Deltaproteobacteria | <i>Geobacter</i> | 100 |
| 9 | 1 | 0 | Unc. <i>Dehalobacter</i> sp. | HM748813 | 99.43 | Clostridia | <i>Acetobacterium</i> | 100 |
| 10 | 4 | 0 | Unc. <i>Sulfurimonas</i> sp. | KF851122 | 98.34 | Epsilonproteobacteria | <i>Sulfuricurvum</i> | 100 |
| 11 | 4 | 0 | <i>Ferribacterium</i> sp. 7A-631 | KF441656 | 99.75 | Betaproteobacteria | <i>Ferribacterium</i> | 93 |
| 12 | 1 | 0 | Unc. Gallionellaceae bacterium | EU266776 | 96.48 | Proteobacteria | Betaproteobacteria | 100 |
| 13 | 1 | 0 | Unc. Rhodocyclaceae bacterium | JQ279024 | 98.83 | Betaproteobacteria | Rhodocyclaceae | 98 |
| 14 | 1 | 0 | Unc. Rhodocyclaceae bacterium | HQ003471 | 97.64 | Betaproteobacteria | Rhodocyclaceae | 100 |
| 15 | 0 | 1 | <i>Acinetobacter baumannii</i> | KI958271 | 99.60 | Gammaaproteobacteria | <i>Acinetobacter</i> | 100 |
| 16 | 0 | 1 | <i>Pseudomonas putida</i> | GU396283 | 98.97 | Gammaaproteobacteria | <i>Pseudomonas</i> | 100 |
| 17 | 0 | 7 | Unc. <i>Bacteroides</i> sp. | AB529592 | 99.44 | Bacteroidia | <i>Parabacteroides</i> | 99 |
| 18 | 0 | 1 | Unc. <i>Bacteroidetes</i> bacterium | FF535139 | 98.31 | Bacteroidia | <i>Parabacteroides</i> | 100 |
| 19 | 0 | 1 | Unc. <i>Bacteroides</i> sp. | JQ624314 | 99.75 | Bacteroidia | <i>Parabacteroides</i> | 100 |
| 20 | 0 | 1 | Unc. <i>Bacteroidetes</i> bacterium | DQ676360 | 98.97 | Bacteroidia | Porphyrimonadaceae | 99 |
| 21 | 0 | 1 | Unc. <i>Bacteroides</i> sp. | FM204969 | 99.88 | Bacteroidia | <i>Parabacteroides</i> | 99 |
| 22 | 0 | 1 | Unc. <i>Acidaminobacter</i> sp. | HM217344 | 98.61 | Clostridia | Clostridiales Incertae Sedis XII | 91 |
| 23 | 0 | 6 | Unc. <i>Acidaminobacter</i> sp. | HM217344 | 98.78 | Clostridia | Clostridiales | 100 |
| 24 | 0 | 5 | Unc. <i>Acidaminobacter</i> sp. | HM217344 | 99.46 | Clostridia | Clostridiales Incertae Sedis XII | 80 |
| 25 | 2 | 0 | Unc. <i>Hydrogenophaga</i> sp. | HM124825 | 99.67 | Clostridia | Clostridiales Incertae Sedis XII | 100 |
| 26 | 3 | 0 | <i>Hydrogenophaga taeniospiralis</i> | AY771764 | 98.02 | Betaproteobacteria | <i>Hydrogenophaga</i> | 100 |
| 27 | 0 | 7 | <i>Malikia spinosa</i> | NR_040904 | 99.86 | Betaproteobacteria | <i>Hydrogenophaga</i> | 100 |
| 28 | 1 | 0 | Unc. <i>Hydrogenophaga</i> sp. | DQ413154 | 98.70 | Betaproteobacteria | <i>Malikia</i> | 100 |
| 29 | 1 | 0 | Unc. Elusimicrobia bacterium | GU236016 | 94.55 | Elusimicrobia | <i>Hydrogenophaga</i> | 98 |
| 30 | 0 | 2 | <i>Hydrogenophaga taeniospiralis</i> | AY771764 | 98.75 | Betaproteobacteria | <i>Elusimicrobium</i> | 95 |
| 31 | 8 | 0 | <i>Hydrogenophaga taeniospiralis</i> | AY771764 | 99.06 | Betaproteobacteria | <i>Hydrogenophaga</i> | 98 |
| 32 | 0 | 6 | <i>Malikia spinosa</i> | NR_040904 | 99.73 | Betaproteobacteria | <i>Malikia</i> | 88 |
| 33 | 1 | 0 | Unc. <i>Acidovorax</i> sp. | AM084039 | 99.04 | Betaproteobacteria | Comamonadaceae | 100 |
| 34 | 3 | 1 | Unc. <i>Dechloromonas</i> sp. | JN679130 | 98.95 | Betaproteobacteria | Rhodocyclaceae | 100 |
| 35 | 1 | 0 | Unc. <i>Gallionella</i> sp. | FJ391502 | 98.72 | Proteobacteria | Betaproteobacteria | 100 |
| 36 | 0 | 2 | <i>Vogesella indigofera</i> | NR_040800 | 99.60 | Betaproteobacteria | <i>Vogesella</i> | 100 |
| 37 | 0 | 1 | <i>Shewanella putrefaciens</i> | JN019028 | 99.87 | Gammaaproteobacteria | <i>Shewanella</i> | 100 |
| 38 | 9 | 0 | <i>Sulfuricurvum kujjense</i> | CP002355 | 99.22 | Epsilonproteobacteria | <i>Sulfuricurvum</i> | 100 |
| 39 | 1 | 0 | Unc. <i>Arcobacter</i> sp. | JQ861849 | 97.96 | Epsilonproteobacteria | <i>Arcobacter</i> | 93 |
| 40 | 2 | 0 | <i>Geobacter metallireducens</i> | NR_075011 | 98.31 | Deltaproteobacteria | <i>Geobacter</i> | 100 |

TABLE 1: Continued.

| OTU | Clones | | Basic Local Alignment Search Tool-GenBank | | CLASSIFIER Match Tool-Ribosomal Database Project II | | % certainty ^A |
|-----|----------------|----------------|---|-----------|---|-----------------------|----------------------------------|
| | t ₀ | t ₁ | Closest described relative | Acc. n° | % identity | Phylogenetic group | |
| 41 | 3 | 0 | Unc. <i>Geobacter</i> sp. | EU266817 | 99.76 | Deltaproteobacteria | <i>Geobacter</i> |
| 42 | 2 | 0 | Unc. <i>Geobacter</i> sp. | EU266841 | 99.16 | Deltaproteobacteria | <i>Geobacter</i> |
| 43 | 5 | 0 | <i>Geobacter lovleyi</i> | NR_074979 | 99.03 | Deltaproteobacteria | <i>Geobacter</i> |
| 44 | 1 | 0 | <i>Geobacter thiogenes</i> | NR_028775 | 97.48 | Deltaproteobacteria | <i>Geobacter</i> |
| 45 | 1 | 1 | Unc. Firmicutes bacterium | HQ003641 | 98.70 | Clostridia | Clostridiales Incertae Sedis XII |
| 46 | 0 | 20 | Unc. Firmicutes bacterium | HQ003641 | 99.45 | Clostridia | <i>Acidaminobacter</i> |
| 47 | 0 | 1 | Unc. <i>Clostridium</i> sp. | FM204998 | 100.0 | Clostridia | <i>Clostridium XIVa</i> |
| 48 | 0 | 3 | <i>Acetobacterium malicum</i> | NR_026326 | 99.53 | Clostridia | <i>Acetobacterium</i> |
| 49 | 1 | 0 | Unc. bacterium | AB759668 | 95.24 | Bacteria | Firmicutes |
| 50 | 2 | 0 | Unc. rumen bacterium | AB615047 | 94.24 | Lentisphaerae | <i>Victivallis</i> |
| 51 | 2 | 0 | Unc. <i>Cytophaga</i> sp. | EU809766 | 99.35 | Lentisphaerae | <i>Victivallis</i> |
| 52 | 1 | 0 | Denitrifying bacterium | FJ802233 | 98.54 | Ignavibacteria | <i>Ignavibacterium</i> |
| 53 | 0 | 8 | Unc. <i>Bacteroides</i> sp. | FJ862827 | 99.18 | Bacteroidia | <i>Parabacteroides</i> |
| 54 | 0 | 3 | <i>Macellibacteroides fermentans</i> | NR_117913 | 99.08 | Bacteroidia | <i>Parabacteroides</i> |
| 55 | 0 | 1 | Unc. Bacteroidetes bacterium | FJ535139 | 94.64 | Bacteroidia | Porphyromonadaceae |
| 56 | 0 | 1 | Unc. Bacteroidetes bacterium | DQ676360 | 99.30 | Bacteroidia | Bacteroidales |
| 57 | 1 | 0 | Unc. <i>Prolixibacter</i> sp. | JQ723616 | 97.85 | Bacteria | Bacteroidetes |
| 58 | 1 | 0 | Unc. <i>Geobacter</i> sp. | JQ086897 | 98.72 | Deltaproteobacteria | <i>Geobacter</i> |
| 59 | 1 | 0 | <i>Geobacter lovleyi</i> | NR_074979 | 99.37 | Deltaproteobacteria | <i>Geobacter</i> |
| 60 | 1 | 0 | <i>Sulfuricurvum kujjense</i> | NR_074398 | 99.29 | Epsilonproteobacteria | <i>Sulfuricurvum</i> |

^AConfidence threshold of the RDP-II CLASSIFIER Tool is 80%.

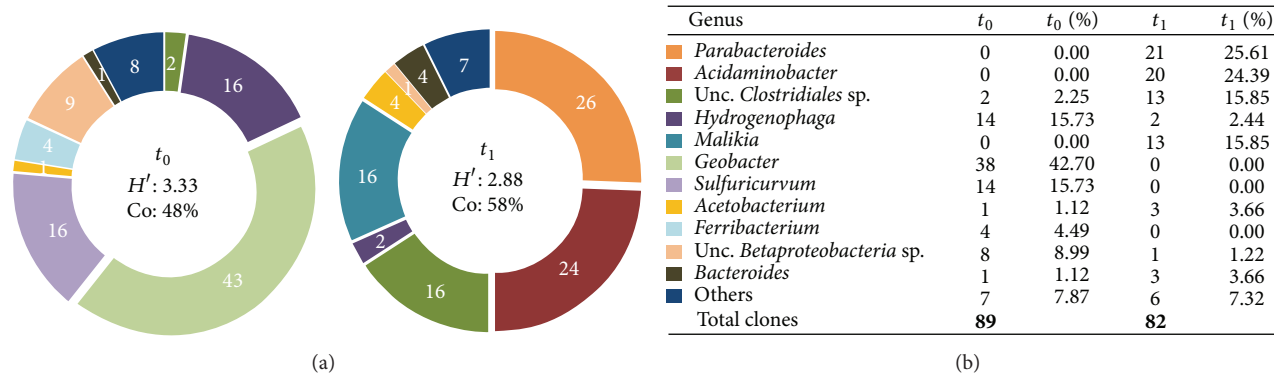


FIGURE 3: (a) Pie charts illustrating the percentages of clones, identified in the bacterial communities at t_0 and at t_1 , grouped in phylotypes at genus level; H' : Shannon Index and Co: community organization (evenness index); (b) table showing the abundance and the percentages of clones grouped in phylotypes at genus level.

microorganisms typically detected in contaminated freshwater ecosystems [40]. For instance, *H. pseudoflava* was identified by Liang and colleagues [41] in a TCE-degrading consortium enriched from TCE-contaminated aquifer sediments and groundwater. A psychrotrophic *H. pseudoflava* strain IA3-A was isolated from polychlorinated biphenyl-contaminated soil and grew on biphenyl as sole carbon and energy source [42]. Both genera, *Hydrogenophaga* and *Sulfuricurvum*, were recently enriched and associated with NO_3^- -reduction in a membrane biofilm reactor inoculated with wastewater sludge and treating perchlorate [43].

The biostimulation with lactate determined a remarkable change of the diversity within the bacterial community. A lower diversity (24 OTUs) was observed and phylotypes related to Firmicutes, Bacteroidetes, and β -Proteobacteria, not detected at t_0 , became dominant; that is, representatives of genera *Acidaminobacter* (20 clones), *Parabacteroides* (21 clones), and *Malikia* (13 clones) were strongly enriched (Table 1, Figure 3). Conversely, *Geobacter*, *Hydrogenophaga*, and *Sulfuricurvum*, the phylotypes dominating the consortium before the treatment, were not detected in the library after the treatment. A similar shift of diversity was previously observed in the upper layer microcosms [9]. However, while in the upper layer of the aquifer phylotypes of known 1,2-DCA dehalogenating genera of the Clostridiales (*Desulfitobacterium* and *Dehalobacter*) were enriched after the biostimulation with lactate, none of the genera enriched in microcosms from the lower layer has been so far associated with reductive dechlorination of 1,2-DCA. Among the phylotypes enriched in the lower layer microcosms, the only characterized representative of genus *Acidaminobacter*, *A. hydrogeniformans*, has been described as a fermentative species whose growth is enhanced by cocultivation with a hydrogen-consuming partner; for example, in our study, it could be a microbe able to couple the H_2 consumption with 1,2-DCA reductive dechlorination [44]. Interestingly, another phylotype enriched at t_1 was related to an uncultured Clostridiales bacterium (12 clones) and, noteworthily, the only reductive dehalogenases specific for 1,2-DCA identified so far were previously associated only with 2 genera

belonging to Clostridiales order: *Desulfitobacterium* [16] and *Dehalobacter* [15]. Taken together, these data indicate that in the lower aquifer the lactate amendment enriched different phylogenetically distant taxa previously not associated with 1,2-DCA dechlorination, suggesting that novel reductive dechlorinators may mediate such a process.

3.2. Reductive Dehalogenase Gene Libraries. The reductive dehalogenase diversity in the lower aquifer was investigated in response to lactate biostimulation to evaluate whether reductive dehalogenating functional redundancy could be associated with the diversity pattern depicted by the *16S rRNA* gene libraries. In previous works, a complete sequence of one RD gene cluster specifically adapted to 1,2-DCA was obtained from microcosms of the upper layer of the aquifer [16]. Three genes (*dcaB*, *dcaC*, and *dcaT*) of the identified RD cluster presented high nucleotide identity (above 98%) with the RDs specific for chlorinated alkenes, but the gene coding for the main catalytic subunit of the reductive dehalogenase (*dcaA*) presented only 94% and 90% nucleotide and amino acid identities. The sequence differences were associated with dechlorination of 1,2-DCA since *Desulfitobacterium dichloroeliminans* strain DCA1, capable of dechlorinating 1,2-DCA but not chlorinated ethene, showed the same amino acid signatures in the two sole RDs identified in the genome [16].

Using the same RD-targeting PCR approach of Marzorati et al. [16], a total of 17 clones were obtained after the treatment, representing four different RDs. Figure 4 shows their phylogenetic relationship with known RDs. The RD sequences found in the lower aquifer layer were grouped in one cluster together with those previously identified in the upper aquifer layer [9]. The percentage of similarity among the newly identified RDs was between 100 and 99% and shared 99% nt identity with WL rdhA1, one of the three RDs identified by Grostern and Edwards [15], in a 1,2-DCA degrading coculture where the main representative was *Dehalobacter* sp. WL. It has been previously shown that the 53% of the total amino acid diversity of *dcaA* RDs (RD-54

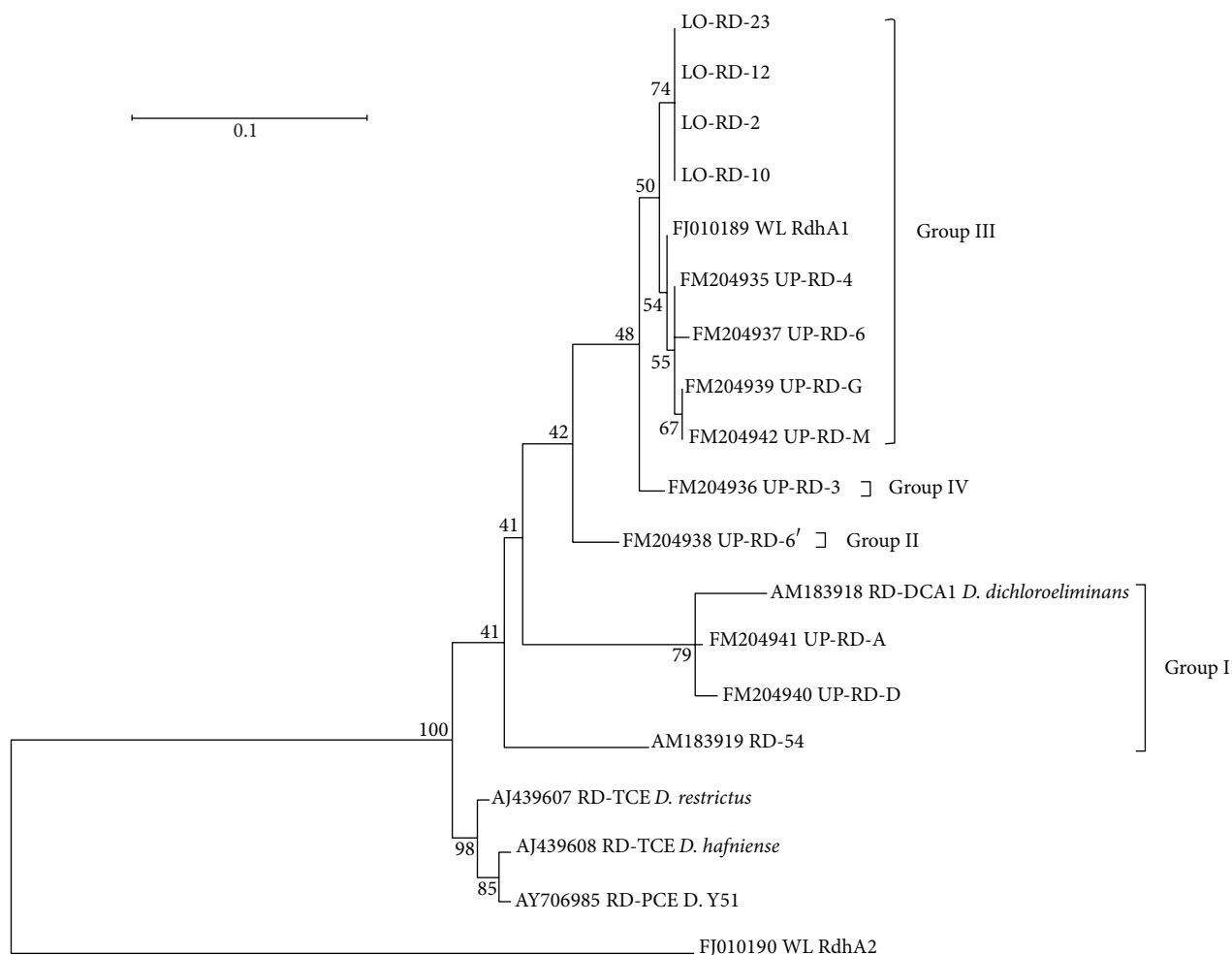


FIGURE 4: Neighbour-joining tree with branch length to assess the relationship between DcaA of the new RDs identified in the lower aquifer (LO-RD-X) and those previously characterized from the upper aquifer (RD-54 [16] and UP-RD-X [9]) and from *D. dichloroeliminans* strain DCA1 (RD-DCA1 [16]). Other A subunits of PceA of *Dehalobacter restrictus* strain DSMZ 9455T (RD-TCE *D. restrictus*: AJ439607), *Desulfotobacterium hafniense* strain TCE1 (RD-TCE *D. hafniense*: AJ439608), *Desulfotobacterium* sp. strain Y51 (RD-PCE *D. Y51*: AY706985), WL rdhA1 (FJ010189), and WL rdhA2 (FJ010190) are also reported. The numbers at each branch point represent percentage of bootstrap calculated from 1000 replicate trees. The scale bar represents the sequence divergence.

and RD-DCA1) with respect to *pceA* RDs specific for tetrachloroethene (PCE; RDs from *Dehalobacter restrictus* strain DSMZ 9455T, *Desulfotobacterium* sp. strain Y51, and *Desulfotobacterium hafniense* strain PCE-S) [12, 45, 46] was mainly localized in two small regions (blocks A and B, Figure 5) that represent only 19% (104 amino acids over 551) of the total *dcaA* residues. These two regions of hypervariability were proposed to be involved in the recognition of 1,2-DCA or in general in the substrate specificity of RDs [16]. The alignment of the RDs identified in the lower aquifer layer with the above-indicated homologs was possible to identify the two mentioned hypervariable regions overlapping with blocks A and B (Figure 5). The alignment permitted identifying amino acids specifically associated with (i) PceA of the PCE-RDs (black residues in a light grey background); (ii) DcaA of group I, specific for WL rdhA1 and for the reductive dehalogenases

enriched from the lower aquifer layer (white residues in a light grey background); (iii) DcaA of group II proposed to be specific for 1,2-DCA RDs from *Desulfotobacterium* (black residues in a dark grey background); (iv) all the RDs within groups I and II but not conserved in the PCE-specific RDs (white residues in a black background).

4. Conclusions

By comparing the diversity of bacteria and RDs in the two aquifer layers following biostimulation with lactate, it can be argued that the RDs linked to 1,2-DCA reductive dechlorination, despite being diverse, are structurally conserved. However, they can be associated with different bacterial carriers selected by the environmental conditions of the specific

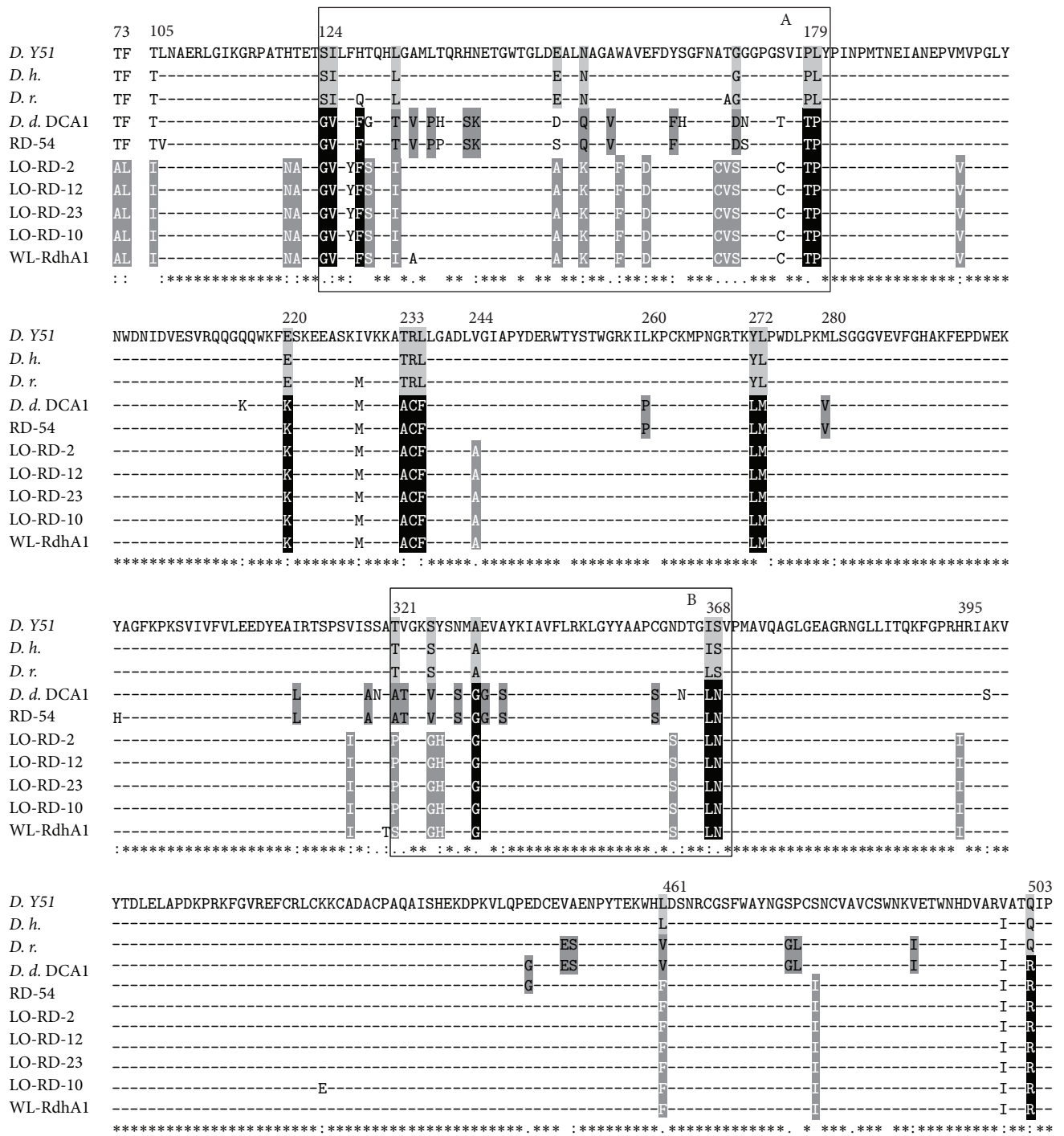


FIGURE 5: Amino acid alignment of the DcaA proteins of the new identified RDs, with those previously identified in the groundwater (RD-54: AM183919) and in *D. dichloroeliminans* strain DCA1 (*D. d. DCA1*: AM183918), with PceA of *Desulfitobacterium* sp. strain Y51 (*D. Y51*: AY706985), *D. hafniense* strain TCE1 (*D. h.*: AJ439608), and *D. restrictus* strain DSMZ 9455T (*D. r.*: AJ439607) and with the WL rdhA1 (FJ010189). Black line (blocks A and B) rectangles indicate two amino acid stretches where 53% of the total amino acid diversity resides between DcaA and PceA [16]. Within blocks A and B of the selected RDs sequences, as well in other smaller regions of the DcaA subunit, it was possible to identify amino acids specific for (i) PceA of the PCE-RDs (black residues in a light gray background), (ii) DcaA of group I, specific for WL rdhA1 and for the reductive dehalogenases identified in the low aquifer after the lactate treatment (white residues in a light gray background), (iii) those of group II proposed to be specific for 1,2-DCA RDs from *Desulfitobacterium* (black residues in a dark gray background), (iv) and finally those common to all the RDs within groups I and II but not conserved in the PCE-specific RDs (white residues in a black background). Asterisks, colons, and dots below the alignment indicate an identical position in all the proteins, a position with a conservative substitution, and a position with a semiconservative substitution, respectively.

aquifer, indicating their plasticity to adapt to different cellular scaffolds and machineries.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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