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Trichloroethene Dechlorination and H₂ Evolution Are Alternative Biological Pathways of Electric Charge Utilization by a Dechlorinating Culture in a Bioelectrochemical System

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Recently, the proof-of-principle of an innovative bioelectrochemical process for trichloroethene (TCE) bioremediation was presented. In this newly developed process, a solid-state electrode polarized to −450 mV (vs SHE), in the presence of a low-potential redox mediator (methyl viologen), is employed as an electron donor for the microbial reductive dechlorination of TCE to lower or nonchlorinated end products. In the present study, we investigated the influence of methyl viologen and TCE concentrations on process performance. Using a highly enriched hydrogenotrophic dechlorinating culture, as a source culture in batch experiments, we found that TCE dechlorination and H₂ evolution were the two main biological reactions which were stimulated. The relative contribution of the two reactions appeared to be strongly dependent on the mediator concentration. At the lowest methyl viologen (MV) concentrations (25–750 μM), only TCE dechlorination was stimulated, and no H₂ was produced; at higher MV concentrations, both reactions occurred simultaneously, although they showed distinct kinetic features. In batch experiments in which TCE was omitted from the system, the rate of H₂ production was remarkably increased (up to 80 times), suggesting that protons represented an alternative electron sink in the absence of the more energetically favorable TCE. Clearly, optimization of the process for TCE dechlorination requires H₂ evolution to be minimized by, for instance, operating the system at low mediator concentrations, and this can be possibly achieved through proper physical immobilization of the mediator at the electrode surface. On the other hand, the observed bioelectrocatalytic H₂ production occurred at virtually no overpotentials with respect to the thermodynamic 2H⁺/H₂ potential. This finding revealed that the dechlorinating culture employed represented quite an exceptional and previously unrecognized biocatalytic system for H₂ production.

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Introduction

The ability of microorganisms to "communicate" with solid-state electrodes by exchanging electrons with them, either directly or via redox mediators (*1*), has recently received considerable attention by the scientific community. This interesting feature is currently being explored as a strategy to produce sustainable electricity (*2–6*) or H₂ (*7–10*) from the bacterial oxidation of organic waste materials in microbial fuel cells or to biologically reduce oxidized pollutants in bioremediation systems (*11–14*). As for the application of bioelectrochemical processes in the field of bioremediation, it was shown that members of the *Geobacter* family biologically reduce nitrate to nitrite or U(VI) to relatively insoluble U(IV) (*13, 14*) using a graphite cathode polarized to −500 mV (vs Ag/AgCl) as the sole electron donor. In *Geobacter*, the extracellular electron-transfer process is mediated by membrane-bound proteins such as cytochromes or highly conductive pili or nanowires (*15, 16*). Some other microorganisms cannot directly transfer electrons to solid electrodes but, rather, require a soluble redox mediator, which serves as an electron shuttle between the cells and the electrodes (*17, 18*). Humic substances, quinones, phenazines, and riboflavin, have been proved to function as exocellular electron mediators (*19, 20*). As an example, Thrash and colleagues showed that washed cells of *Dechloromonas* and *Azospira* species readily reduced 90 mg/L perchlorate, with a solid-state electrode as an electron donor, with 2,6-anthraquinone disulfonate (AQDS) as a mediator (*12*); no perchlorate was reduced in the absence of cells or AQDS, or in an open-circuit control.

In a recent paper, we described the proof-of-principle of a novel process named BEARD (i.e., Bio-Electrochemically Assisted Reductive Dechlorination) in which a polarized glassy carbon electrode (i.e., cathode), in the presence of methyl viologen (MV), as a redox mediator, is used as an electron donor for the microbial reductive dechlorination of trichloroethene (TCE), a common groundwater pollutant, to harmless ethene (*21*). In the BEARD process, the mediator, either dissolved in the bulk liquid or physically "anchored" to the electrode surface, facilitated the shuttling of electrons from the carbon electrode to the dechlorinating bacteria. The proposed process potentially carries several advantages over traditional bioremediation processes for chlorinated solvents which rely on the use of chemicals as electron donors in the bacterial metabolism. Among them are the possibility of continuously monitoring the microbial activity and controlling the supply of electron donors both in terms of current and cathode potential; no chemicals need to be added for electron supply, which omits the need for transport, storage, dosing, post-treatment, and secondary groundwater contamination phenomena (*22, 23*).

The aim of the present study was to gain a deeper insight into the factors affecting the performance of the BEARD process and the mechanisms of electron transfer in a mixed dechlorinating culture. By investigating the influence of redox mediators and TCE concentration, we discovered that proton reduction to molecular H₂ represents an alternative route of electron utilization.

Experimental Section

Source Culture. An anaerobic TCE dechlorinating culture was used as the source culture in bioelectrochemical batch experiments. The culture was originally enriched from brackish sediments originating from the Venice Lagoon (*24*). The culture was maintained in a 2 L fill-and-draw bioreactor

which consisted of a continuously stirred borosilicate glass bottle (liquid volume 1.5 L), sealed by a Teflon-faced gray-butyl stopper (Wheaton, Millville, NJ) and crimped by an aluminum cap. The reactor was fed once per week with TCE (0.75 mmoles) and H_2 (1.64 mmoles). Before each refeeding, the reactor was flushed with anaerobic gas (70:30 N_2/CO_2) to remove volatile compounds. On average, 450 mL of suspended culture was weekly removed from the reactor and used in bioelectrochemical batch experiments (the resulting average cell retention time was 20−25 days). The removed volume was weekly replaced by a fresh anaerobic basal medium, which contained (g/L): $(NH_4)_2Cl$, 0.5; $MgCl_2 \cdot H_2O$, 0.1; K_2HPO_4, 0.4; $CaCl_2 \cdot H_2O$, 0.05; 10 mL of a trace metal solution (25); 1 mL of a vitamin solution (26); and 15 mL of $NaHCO_3$ (10% w/v). The pH of the medium was 7.5. The reactor was maintained in a water bath at 25 ± 1 °C. After several feeding cycles, the culture could steadily dechlorinate TCE at a maximum rate of 255 ± 19 μeq/day. The spiked TCE was dechlorinated to predominantly cis-dichloroethene (cDCE), although lower amounts of vinyl chloride (VC) and ethene (ETH) were also formed. No methane was ever produced in the culture. The steady-state biomass concentration was around 20−25 mg VSS/L. A mass balance of reducing equivalents in the reactor indicated that typically over 90% of the added hydrogen was typically recovered as TCE dechlorination products. A molecular analysis of the culture by fluorescent in situ hybridization (FISH) carried out using previously developed probes and protocols (27, 28) indicated that *Desulfitobacterium* sp. (i.e., a known H_2-utilizing, TCE-to-cDCE dechlorinating bacterium) (29, 30) was the predominant (58.4 ± 5.7% Dsf440/EUB338mix) member of the microbial community. Although in lower percentages (11.8 ± 2.8% Dhe1259t-c/EUB338mix), *Dehalococcoides* spp. was also detected in the reactor. FISH analysis also confirmed the absence of methanogenic populations. Epifluorescence micrographs and additional information on the characterization of the microbial culture by FISH can be found in the Supporting Information. It is worth mentioning that, when the same source culture was operated at a higher cell retention time (52 days), the composition of the community was remarkably different, with *Dehalococcoides* spp. being the dominant member (21).

Bioelectrochemical Cell Setup. The bioelectrochemical cell used in this study consisted of two gastight borosilicate glass bottles (total volume 250 mL) separated by a 3 cm^2 cross-sectional area, a Nafion 117 (DuPont) proton exchange membrane (PEM). The PEM was pretreated by boiling in H_2O_2 (30%), then in 0.5 M H_2SO_4, and finally in DI water, each for 1 h, and then stored in DI water prior to being used. Each bottle was equipped with side arms for the sampling of the headspace (total volume 100 mL) and the liquid phase (total volume 150 mL), which ended with Teflon-faced butyl rubber stoppers and aluminum crimps. The cathode we used was a glassy carbon rod (HTW GmbH, Germany; 5 mm diameter, 150 mm length, working surface area ~7 cm^2), and the anode was a 2 cm^2 platinum disk. The electrodes were repeatedly soaked in DI water prior to being used. The reference electrode (placed in the cathode compartment) was a saturated Ag/AgCl electrode (+199 mV vs SHE; Amel S.r.l., Milano, Italy). Electrochemical measurements and monitoring were performed using a Galvanostat/Potentiostat MACCOR 4000 (Kyunggi-Do, Korea) interfaced to a PC. A VMP potentiostat (Bio-Logic, Grenoble, France) was used for cyclic voltammetry experiments, which were performed in a reduced basal medium (see below) as the supporting electrolyte.

Batch Dechlorination Experiments. For bioelectrochemical batch experiments, 150 mL of the source culture was anaerobically transferred into the cathode compartment of the bioelectrochemical cell using a glass syringe. In parallel,

the anode compartment was filled with an identical volume of a mineral medium. Thereafter, the two compartments were flushed with anaerobic gas (70:30 N_2/CO_2). After an equilibration period (~1 h), the cathode compartment was spiked with the desired volume of TCE and/or a solution of MV; cyclic voltammograms were recorded to characterize the electrochemical activity of the redox mediator, and then the cathode potential was set to −450 mV (vs SHE). At regular intervals, gaseous samples (100−500 μL) were removed from the headspace of the compartments, using gastight, sample-lock Hamilton (Reno, NV) syringes, and analyzed by gas chromatography for volatile compounds. Control experiments were also performed in the absence of TCE, or in the absence of the microbial culture, or in the absence of cathode polarization.

In all tests, the bioelectrochemical cell was maintained at 25 °C in a water bath under magnetic stirring. The cumulative electric charge (μeq) that was transferred at the electrodes was calculated by integrating the current (A) over the period of electrode polarization. Cumulative reducing equivalents (μeq) that were used for reductive dechlorination were calculated from the measured amounts of TCE dechlorination products formed, considering that 2 μeq/μmol are required for the removal of each chlorine. Hydrogen formed was also expressed as reducing equivalents (μeq) using 2 μeq/μmol as molar conversion factor.

Analytical Methods. Volatile compounds (TCE, cDCE, VC, ETH, and methane) were analyzed in 100 μL gaseous samples, using a gas chromatograph Varian 3400 (Palo Alto, CA) equipped with a flame ionization detector, as described previously (31, 32). H_2 was analyzed in a 500 μL gaseous sample by a Trace Analytical TA3000R reduction gas detector (RGD; Menlo Park, CA). When the H_2 level was above the range of the RGD (i.e., >0.5 μM), it was quantified using a Varian 3400 gas chromatograph and a thermoconductivity detector (32). Headspace, concentrations were converted to aqueous-phase concentrations using tabulated Henry's law constants (33, 34).

Chemicals. Methyl viologen was purchased from Sigma-Aldrich as the dichloride salt and was used as received. Neat TCE (99.5+ %) was purchased from Sigma-Aldrich. Vinyl chloride, ethene, ethane, hydrogen, and methane gases (99.9+ %) were purchased from Scott Specialty Gases (Bellefonte, PA). All of the other chemicals used to prepare analytical standards or feed solutions were of analytical grade and were used as received.

Results

BEARD at −450 mV (vs SHE) with Dissolved MV. In order to achieve significant rates of electron transfer between the glassy carbon electrode and the dechlorinating bacteria, the BEARD process required the presence of a low-potential (E° = −446 mV vs SHE) redox mediator (i.e., MV). Indeed, as shown in Figure 1, negligible dechlorination of the spiked TCE (15 μmol) occurred, in batch experiments, when either the dechlorinating culture or MV was omitted from the system or when no polarization was applied to the cathode (test types 1−3; Figure 1A). Conversely, an almost linear accumulation of TCE dechlorination products (i.e., cis-DCE and lower levels of VC) occurred under typical BEARD conditions (test type 4), that is, in the presence of the dechlorinating culture, MV (500 μM), and cathode polarization to −450 mV (vs SHE). At the end of the test, the amount of produced cis-DCE, on a molar basis, was approximately 10 times more than the amount of produced VC.

Since H_2 is an important electron donor in the microbial reductive dechlorination of TCE, the H_2 liquid-phase concentration during the above-described tests was monitored and is shown in Figure 1B. In control experiments (test types 1−3), the H_2 concentration remained nearly constant at
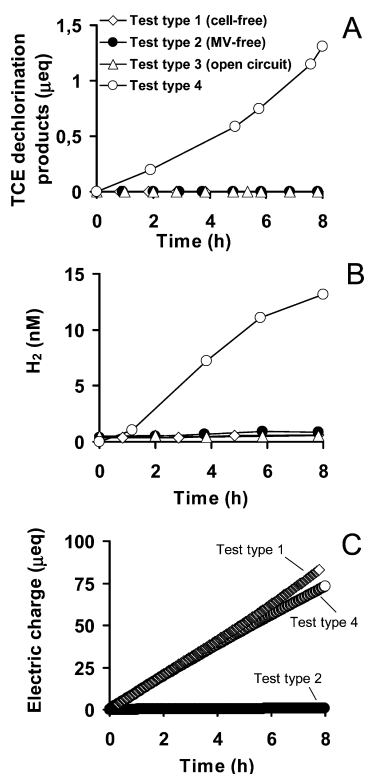
FIGURE 1. Reducing equivalents channeled to TCE dechlorination (A) and liquid-phase H_2 concentration (B), in batch experiments, and cumulative electric charge transferred (C). Test type 1 (cell-free): abiotic batch experiment with TCE (15 μmoles), MV (500 μmol/L), cathode polarization (-450 mV vs SHE), but without the microbial culture. Test type 2 (MV-free): biotic batch experiment with TCE (15 μmol), the microbial culture; cathode polarization (-450 mV vs SHE), but without MV. Test type 3 (open circuit): biotic batch experiment with TCE (15 μmoles), MV (500 μmol/L), the microbial culture, but without cathode polarization. Test type 4: biotic batch experiment with TCE (15 μmoles), MV (500 μmol/L), cathode polarization (-450 mV vs SHE), and the microbial culture.

around 0.5 nM, confirming that H_2 could not be produced from the abiotic electrochemical proton reduction. Analogously, the endogenous metabolism of the microbial suspension also resulted in a negligible accumulation of H_2. By contrast, H_2 accumulated at -450 mV (vs SHE), in the presence of the microbial culture and MV (500 μM), even though at a very low rate (0.7 μeq/day) and concentration ($\sim$15 nM). This finding proved that electrically reduced methyl viologen could act as a reductant for a biologically mediated reduction of protons to H_2.

During type 1 and type 4 batch experiments (i.e., in the presence of MV and cathode polarization), the current flowing in the circuit was nearly the same ($\sim$250 μA) and remained constant throughout the test, independently of the presence of the microbial culture. Conversely, in the absence of MV (test type 2), the current remained always below 5 μA. Accordingly, the cumulative electric charge transferred during the 8 h of the test was nearly the same for type 1 and type 4 batch experiments, and almost negligible for type 2 batch experiments (Figure 1C). Therefore, under the experimental conditions adopted for test type 4, only a low percentage of the electric charge could be channeled to the slow dechlorination and H_2 production. The steady-state current reached in the presence of MV (regardless of the presence of the microbial culture and dechlorinating activity) was presumably controlled by the rate of the reaction between electrochemically reduced MV with the oxygen generated at the anode (from water oxidation) and diffusing into the cathode
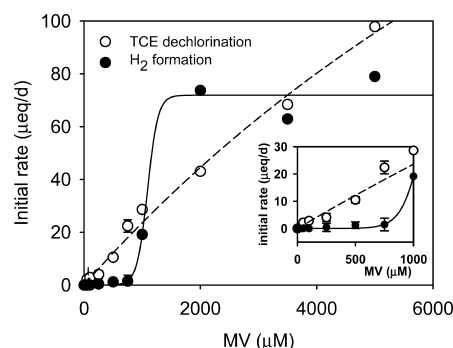

FIGURE 2. Influence of MV concentration on the initial rate of TCE dechlorination (open circles) and H_2 formation (filled circles) by the microbial culture in batch experiments at -450 mV (vs SHE). Error bars represent the standard deviation of replicated experiments.

chamber through the Nafion membrane. Under such experimental conditions, we could reasonably assume that the concentration of the reduced form of MV remained nearly constant throughout each test. Thus, since the maximization of current efficiency was not, at this stage, the final aim of these tests, it was considered that the latter condition (constant concentration of the reduced form of MV at steady-state current) was favorable to investigate the role of MV on the different biological reactions.

Influence of MV Concentration on TCE Dechlorination and H_2 Formation. Figure 2 shows the influence of MV concentration on the initial rate of TCE-dechlorination and H_2 formation by the mixed culture, with the cathode polarized at -450 mV (vs SHE). Each data point in the graph shown in Figure 2 resulted from an independent batch experiment. In the range of MV concentration 25–5000 μM, the initial TCE dechlorination rate increased in nearly direct proportion to MV concentration (Figure 2). In contrast, at MV concentrations of 7500 μM (data not shown), the initial rate of TCE dechlorination (and also H_2 formation) was significantly lower than at 5000 μM, indicating that such a high MV concentration proved toxic to microorganisms or that dimerization reactions of the radical $MV^{+\bullet}$ (i.e., the reduced form of MV) resulted in a decreased bulk concentration of the biologically active form of the redox mediator.

Compared to TCE dechlorination, the rate of H_2 formation showed a remarkably different dependency on MV concentration. More specifically, no (or very slow) H_2 production was observed in the range of MV concentrations from 25 to 750 μM; a sharp, 100-fold, increase in the H_2 formation rate was observed in the range of MV concentrations from 750 to 2000 μM; then, at higher MV concentrations, the H_2 formation rate did not increase further (Figure 2).

It is worth mentioning that control experiments carried out over the same range of MV concentrations (i.e., 25–5000 μM) confirmed that neither was H_2 produced nor was TCE dechlorinated unless microorganisms, MV, and cathode polarization (at -450 mV vs SHE) were simultaneously present in the system (data not shown).

Influence of TCE on H_2 Formation. Batch experiments at three different MV concentrations (i.e., 250, 750, and 3500 μM) were conducted also in the absence of TCE. As shown in Figure 3, also in the absence of TCE, the rate of H_2 formation showed an apparent saturation dependency on the MV concentration: it sharply increased (from 6.8 μeq/day to 65.2 μeq/day) when the added MV increased from 250 to 750 μM, then almost leveled off (at a value of around 80 μeq/day) when the MV concentration was further increased to 3500 μM.

The most striking finding that emerged from the couples of batch experiments with and without TCE, at different MV concentrations, is that the initial rate of H_2 formation in the
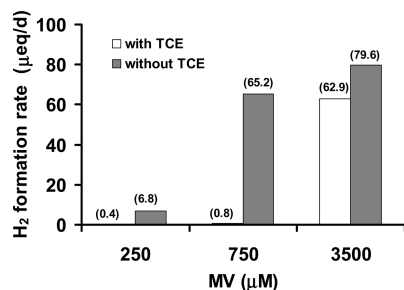
FIGURE 3. Influence of MV concentration on the initial H_2 formation rate (values are indicated in parentheses) by the microbial culture with or without TCE (15 μmol). All of the experiments were performed with the cathode polarized to −450 mV (vs SHE).
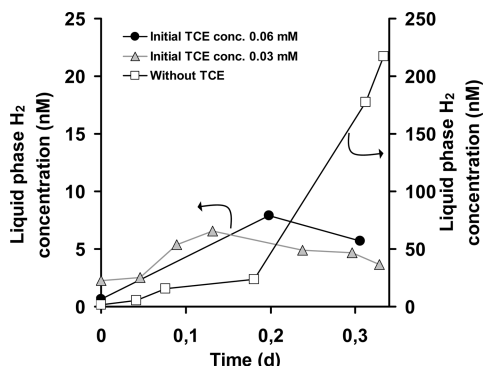

FIGURE 4. Time course of H_2 liquid-phase concentration during batch experiments in the presence of different initial TCE concentrations.

absence of TCE was much greater than in the presence of TCE. Such an enhancement was observed at all of the tested MV concentrations, although it was maximum (~80 times) when the MV concentration was 750 μM (Figure 3).

To verify whether the higher H_2 formation rate was due to the absence of inhibitory effects of TCE on H_2-producing microorganisms, additional tests with lower amounts of spiked TCE were conducted. In these tests, the initial liquid phase concentration of TCE was decreased from 60 μM down to 30 μM (Figure 4), while the MV concentration was maintained at 250 μM. In spite of the diminished initial TCE concentration, no differences were observed among the observed H_2 profiles, suggesting that toxicity effects were unlikely the reason for the reduced H_2 formation in the presence of TCE (Figure 4). Figure 4 also shows the time course of H_2 concentration when TCE was completely omitted from the system: at the end of the 8 h experiment, the H_2 concentration was over 1 order of magnitude higher than in the experiments with various initial amounts of TCE.

Interestingly, at the highest MV concentrations, both in the presence and in the absence of TCE, the H_2-formation rate reached almost the same value of around 80 μeq/day, thereby suggesting that this value represented a maximum rate of this reaction, for the microbial culture under consideration. In support of this consideration, thermodynamic calculations indicated that, at the highest H_2 partial pressures reached (e.g., 0.0025 atm), for the reaction to be energetically feasible, it was sufficient that only 1/30 of added MV was in its reduced state. Accordingly, at the highest MV concentrations, H_2 production was most probably limited by the intrinsic kinetic activity of the culture rather than by thermodynamic constraints.

Discussion

Relevance of H_2 Evolution on BEARD Efficiency.
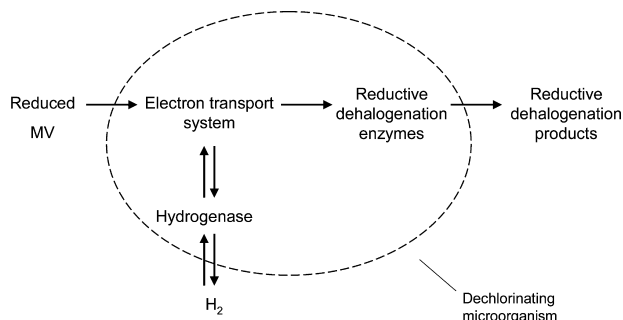This study revealed that TCE dechlorination and H_2 evolution are the

FIGURE 5. Proposed scheme of reduced MV utilization, TCE dechlorination, and H_2 production by dechlorinating bacteria (modified from ref 35).

two main biological reactions which are stimulated in the BEARD process when a mixed, hydrogenotrophic, TCE-dechlorinating culture is employed. The relative importance of the two reactions appeared to be strongly dependent on the mediator concentration. At the lowest MV concentrations (25−750 μM), only TCE dechlorination was stimulated, whereas at higher MV concentrations, both reactions occurred simultaneously, although they showed distinct kinetic features. Although in principle, H_2 is also an electron donor for TCE dechlorination, its accumulation in the system actually represents a source of current and process inefficiency (e.g., other metabolisms can be stimulated causing the formation of unwanted end products such as methane), which thereby needs to be minimized by operating the system at low mediator concentrations. Clearly, this would also result in lower degradation rates. Possibly, a combination of high degradation rates and high current efficiency could be obtained by anchoring the mediator at the electrode surface and employing high specific surface electrodes. Indeed, in a previous study (21), we found that, using a glassy carbon electrode with the mediator immobilized in a Nafion film on its surface, high current efficiencies were obtained without hydrogen accumulation. At the same time, the use of high specific surface area electrodes should, in principle, also allow the achievment of high bacterial densities in the system and accordingly high dechlorination rates.

Putative Microbial Populations Responsible of H_2 Formation and Reductive Dechlorination. The results obtained in this study suggest two alternative hypotheses concerning the microbial populations responsible for H_2 formation and TCE dechlorination. In a first hypothesis, H_2 production and TCE dechlorination are catalyzed by distinct microbial populations which both use electrochemically reduced MV as an electron donor in their metabolism. The dechlorinating bacteria, in addition to MV, also use the H_2 produced by other community members as an electron donor for TCE dechlorination. According to this hypothesis, the apparent lack of H_2 formation at the lowest MV concentrations (25−750 μM) could actually be explained by a rapid scavenging of the slowly produced H_2 by dechlorinators. In a second, and we believe more convincing, hypothesis, the observed H_2 production would result from a branched metabolism of the dechlorinating bacteria themselves (Figure 5): when electron donation by reduced MV exceeds electron utilization by TCE reductive dehalogenation enzymes (this occurs at increasing MV concentrations), the electrons are diverted, most likely via a hydrogenase, to an alternative electron sink, that is, H^+. However, at the lowest MV concentrations and in the presence of TCE, the H_2-producing pathway is not active because the electrons are channeled to the most energetically favorable electron acceptor (i.e., TCE). Alternatively, in the absence of TCE, all of the electron flow is diverted toward protons' reduction, and the H_2 formation is greatly enhanced. It is worth noting that the latter strategy has been previously

described for a sulfate-reducing microorganism using metallic iron as a direct source of electrons via an extracellular pathway (*35*). Similarly to sulfate-reducing bacteria, also dechlorinators, including those in our mixed culture, can use H_2 as an electron donor in their metabolism. In these bacteria, the H_2 metabolism involves key-enzymes, hydrogenases, which are known to catalyze the reversible reaction $H_2 \leftrightarrow 2H^+ + 2e^-$ (*36*). In support of this hypothesis is also the fact that TCE dechlorination was apparently the only metabolism responsible for H_2 consumption in the present mixed culture used as inoculum in the batch experiments. Indeed, no methane was ever detected, and regularly, nearly all of the weekly fed H_2 was recovered as TCE dechlorination products (data not shown). Moreover, the microbiological characterization of the mixed culture by FISH revealed that known hydrogenotrophic dechlorinating microorganisms (*Desulfitobacterium* spp. and *Dehalococcoides* spp.) accounted for over 70% of the total bacteria.

Relevance of Biocathodic H_2 Production. The interest in hydrogen evolution catalysts is currently increasing, as molecular hydrogen is being considered as an optimal energy carrier. Hydrogenases are very effective catalysts for the hydrogen evolution process (*37, 38*); therefore, hydrogenase-anchored electrodes aimed at exploiting the interesting catalytic features of these enzymes are currently being developed (*39, 40*). However, these systems suffer from the fact that enzymes are typically instable and are not self-regenerating. Conversely, the bioelectrocatalytic H_2 production by self-regenerating whole cells (instead of enzymes) would be more appealing. So far, bioelectrocatalytic H_2 production has been reported only for an immobilized pure culture of *Desulfovibrio vulgaris* (which contains periplasmic hydrogenases) with MV as a redox mediator (*38, 41*). Only very recently, Rozendal and colleagues (*42*) developed, for the first time, a H_2-producing biocathode based on a mixed culture of "electroactive" bacteria able to directly accept electrons from a polarized electrode, via an extracellular electron transfer pathway. However, the composition of the microbial culture described in that study was unknown. A preliminary comparison of the maximum volumetric H_2 production rate obtained in this study with those reported by Rozendal and colleagues (*42*) indicates that rates here obtained are about 100 times lower. However, it needs to be considered that the biomass concentration in batch experiments was extremely low and typically around 20 mg VSS/L. Accordingly, it is anticipated that H_2 production rates comparable to those reported by Rozendal and colleagues (*42*) should be potentially achieved by employing high surface area electrodes, which, in the end, should allow an increase of the bacterial density by orders of magnitude. Overall, our findings revealed that a dechlorinating culture (and most likely hydrogenotrophic dechlorinating bacteria) is quite an exceptional biocatalyst for H_2 production, exhibiting almost no overpotentials with respect to the thermodynamic $2H^+/H_2$ potential (-440 mV vs SHE, pH = 7.5). Although promising, the practical applicability of such microorganisms for sustainable bioelectrocatalytic H_2 production will surely require further investigations, including a long-term assessment of culture viability and stability of the redox mediator.

Acknowledgments

Supporting Information Available

Epifluorescent micrographs and information on the molecular characterization of the dechlorinating culture by FISH. This material is available free of charge via the Internet at http://pubs.acs.org.

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