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Effects of Electron Donor and Acceptor Conditions on Reductive Dehalogenation of Tetrachloromethane by *Shewanella putrefaciens* 200

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***Shewanella putrefaciens* 200 is a nonfermentative bacterium that is capable of dehalogenating tetrachloromethane to chloroform and other, unidentified products under anaerobic conditions. Since *S. putrefaciens* 200 can respire anaerobically by using a variety of terminal electron acceptors, including NO_3^-, NO_2^-, and Fe(III), it provides a unique opportunity to study the competitive effects of different electron acceptors on dehalogenation in a single organism. The results of batch studies showed that dehalogenation of CT by *S. putrefaciens* 200 was inhibited by O_2, 10 mM NO_3^-, and 3 mM NO_2^-, but not by 15 mM Fe(III), 15 mM fumarate, or 15 mM trimethylamine oxide. Using measured O_2, Fe(III), NO_2^-, and NO_3^- reduction rates, we developed a speculative model of electron transport to explain inhibition patterns on the basis of (i) the kinetics of electron transfer at branch points in the electron transport chain, and (ii) possible direct inhibition by nitrogen oxides. In additional experiments in which we used 20 mM lactate, 20 mM glucose, 20 mM glycerol, 20 mM pyruvate, or 20 mM formate as the electron donor, dehalogenation rates were independent of the electron donor used. The results of other experiments suggested that sufficient quantities of endogenous substrates were present to support transformation of tetrachloromethane even in the absence of an exogenous electron donor. Our results should be significant for evaluating (i) the bioremediation potential at sites contaminated with both halogenated organic compounds and nitrogen oxides, and (ii) the bioremediation potential of iron-reducing bacteria at contaminated locations containing significant amounts of iron-bearing minerals.**

Although they are resistant to aerobic biodegradation, heavily halogenated organic compounds, including tetrachloromethane (CT), can often be dehalogenated under anaerobic conditions. It has recently been shown that in addition to dehalogenation by strictly anaerobic bacteria, CT can be dehalogenated by facultatively anaerobic, iron-reducing bacteria (18). Such bacteria may be important in environmental dechlorination processes since conditions in many contaminated sediments (e.g., intermittent aerobic-anaerobic conditions and the presence of iron-containing minerals) may favor these microorganisms. Since different electron acceptors, such as nitrate, may be present as cocontaminants in such environments, it is important to study the effects of different electron acceptors on dehalogenation.

Previous studies of reductive dehalogenation in which mixed cultures were used established that the dehalogenation rate, extent, and pattern are often determined by the prevailing electron acceptor conditions (1, 5, 10, 11) or the electron donor present (4, 15). When mixed cultures are used, however, changes in dehalogenation activity may be due to succession in the bacterial community that results from different electron acceptor conditions.

There have been few studies in which dehalogenation by pure cultures under different electron acceptor conditions has been examined. Linkfield and Tiedje found that sulfoxy anions inhibited dechlorination of chlorobenzoate by *Desulfomonile tiedjei*, whereas nitrate and fumarate did not (13). Using resting-cell suspensions of the same organism, DeWeerd et al. found that sulfite and thiosulfate inhibited dehalogenation of 3-chlorobenzoate and suggested that a mutual electron carrier was involved in both reductive dehalogenation and reduction of sulfoxy anions (8). Criddle et al. studied the ability of *Escherichia coli* K-12 to transform CT under different electron acceptor conditions (7). Transformation was greatest in fumarate-respiring and fermentative cultures and was inhibited by the presence of nitrate or under highly aerobic conditions. No effort was made to provide a mechanistic explanation for the different rates of transformation.

Shewanella putrefaciens 200 is a nonfermentative bacterium that is capable of dehalogenating CT to chloroform and other, unidentified products under anaerobic conditions (18). Since *S. putrefaciens* 200 can respire anaerobically by using NO_3^-, NO_2^-, Fe(III), and trimethylamine oxide (TMAO), it provides a unique opportunity to study the competitive effects of different electron acceptors on dehalogenation rates in a single organism. In this paper we describe (i) the effects of various electron donors and electron acceptors on rates of CT transformation by *S. putrefaciens* 200 and (ii) a speculative model to explain the results observed.

Several electron donors (glucose, lactate, formate, pyruvate, and glycerol) were added during stationary-phase dehalogenation experiments in which an electron acceptor was not present. The electron acceptors tested in additional experiments were oxygen, Fe(III), nitrate, nitrite, and TMAO. Since the results of previous work (2, 3, 14) suggested that branched electron transport pathways are developed by *S. putrefaciens* 200 during growth under low oxygen tensions, we conducted experiments with cultures grown under both highly aerobic and microaerobic conditions.

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MATERIALS AND METHODS

Bacterial strain and growth conditions. *S. putrefaciens* 200 was grown as previously described (18) under either highly aerobic ([O_2], $>2.0 \times 10^{-4}$ M) or microaerobic ([O_2], $<2.5 \times 10^{-6}$ M) conditions in a Biostat MD fermentor (B. Braun Biotech). Unless indicated otherwise, cultures were grown at 30°C on Westlake medium (16) containing 18 mM (initial concentration) lactate as the carbon source.

Rates of electron transport to different electron acceptors. The rates of electron transport to a series of electron acceptors, including O_2, Fe(III), nitrate, and nitrite, were determined in batch cultures. Cells were grown under highly aerobic and microaerobic conditions to optical densities at 600 nm of 0.370 and 0.900, respectively. The cells were harvested by centrifugation and resuspended in fresh Westlake medium at 23°C. Nitrate and nitrite were added from autoclaved stock solutions of KNO_3 and $NaNO_2$. The Fe(III) stock solution was prepared as an aqueous ferric citrate solution and was filter sterilized before it was added. The initial concentrations of NO_3^-, NO_2^-, and Fe(III) were 5.0, 3.0, and 3.8 mM, respectively. The rates of electron transport were estimated from the time-dependent concentrations of NO_3^-, NO_2^-, and Fe(II).

General protocol for dehalogenation experiments. Aerobically or microaerobically grown cells were harvested by centrifugation, resuspended in fresh medium to experiment-dependent cell densities, and dispensed into 72-ml serum bottles (60 ml per bottle) as previously described (18). Unless indicated otherwise, dehalogenation experiments were performed in the absence of a physiological electron acceptor. The bottles were purged with oxygen-free nitrogen gas and sealed with Teflon-coated stoppers before CT was added.

In experiments in which we examined the effects of different electron donors, lactate-grown cultures were washed twice and resuspended in medium lacking a physiological electron acceptor but containing 20 mM lactate, 20 mM glucose, 20 mM glycerol, 20 mM pyruvate, or 20 mM formate. Autoclaved cultures containing 18 mM lactate were used as controls. Additional controls were prepared by resuspending microaerobically grown cells in medium lacking any carbon source; Westlake medium without lactate and yeast extract was used for this. The substrate-free suspension was mixed at 23°C for 2 h under aerobic conditions in an effort to remove residual substrate and to deplete intracellular reserves before the "starved" cell suspension was dispensed into serum bottles (60 ml per bottle). Resazurin (0.0001%) was added to the starved cell controls as a redox indicator. CT was added after the headspace was purged with nitrogen gas. Two bottles in each set were prepared with an air headspace.

For experiments in which we studied dehalogenation in the presence of different electron acceptors, sterile aqueous stock solutions of KNO_3, $NaNO_2$, fumarate, and TMAO were prepared so that a 1.0-ml addition to 59 ml of culture would result in final concentrations of 10, 3.0, 15, and 15 mM, respectively. Fe(III) was added from a sterile stock solution of ferric citrate to a final concentration of 15 mM unless indicated otherwise.

In the experiments in which we studied the effects of TMAO, an additional replicate bottle was used to monitor the changes in pH that accompanied TMAO reduction to trimethylamine. HCl was added periodically to TMAO-containing bottles to maintain pH values within the range from 6.8 to 7.2. This process typically resulted in addition of 0.2 to 0.3 ml of 2.0 N HCl to each 60-ml liquid culture over the course of an experiment.

CT was added to sealed serum bottles by injecting 5 μl of a methanol-based CT stock solution, which resulted in an aqueous phase CT concentration of 19.5 μM (3 ppm). The bottles were wrapped in aluminum foil and incubated horizontally on a shaker table (150 to 250 rpm) at 22 to 23°C. CT transformation was then monitored over a 10- to 14-day period as described below. All experiments included two or three controls containing sterile water or autoclaved cultures spiked with CT. The losses in the sterile-water controls were identical to the losses in poisoned or autoclaved cultures (data not shown).

Analytical methods. Fe(II) concentrations were determined by using either a ferrozine-based method derived from the method described by Arnold et al. (3) or a 1,10-phenanthroline colorimetric assay. Both ferrozine and 1,10-phenanthroline were obtained from Sigma Chemical Co. Total iron concentration was determined following reduction with a few grains of hydroxylamine hydrochloride. Oxygen utilization rates were measured with a model 5300 biological monitor (Yellow Springs Instrument Co., Inc.). Nitrate and nitrite concentrations were analyzed with a Dionex QIC analyzer equipped with a Dionex IonPac column (type HPIC-AS4A) and a Dionex IonPac A64A guard column.

CT and chloroform concentrations were determined by gas chromatography, using headspace samples as previously described (18) or following pentane extraction. The Coomassie staining method of Bradford (6) was used to measure total protein contents in cell extracts prepared as described elsewhere (18).

RESULTS AND DISCUSSION

Electron donor effects. The rates of stationary-phase CT transformation, (i.e., CT transformation rates without a physiological electron acceptor) were similar regardless of which electron donor (lactate, glucose, formate, pyruvate, or glycerol) was provided (data not shown). Substrate-free, starved cultures of *S. putrefaciens* were also able to transform CT at similar rates when anoxic conditions were initially established through the use of a nitrogen headspace. Resazurin was decolorized in the starved-cell reactor within 1 h after the contents were purged with dinitrogen gas, indicating that endogenous, cellular reserves were sufficient to establish reducing conditions. However, CT dehalogenation in starved-culture bottles that were sealed with an air headspace was delayed until the oxygen in the headspace was depleted and resazurin was decolorized (data not shown). This lag period was shortened by the presence of lactate.

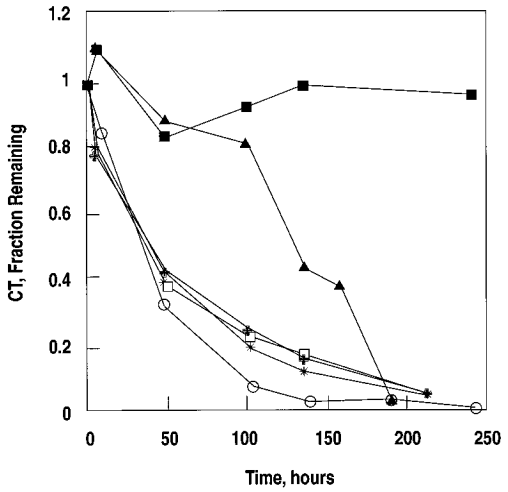

FIG. 1. Dehalogenation of CT by microaerobically grown cultures of *S. putrefaciens* 200 in the presence of various electron acceptors. The electron acceptors used were nitrate (▲), TMAO (○), Fe(III) citrate (*), and fumarate (+); we also included a culture containing no electron acceptor (□) and a sterile water control (■). Each electron acceptor was added at a concentration of 15 mM. The substrate provided was 18 mM lactate. The values are the means of the values obtained for cultures in two replicate bottles. The initial mass of CT used was 1.44 μmol (aqueous concentration, 3 ppm). The initial culture optical density at 600 nm was 1.51.

Under the experimental conditions described above, *S. putrefaciens* apparently possessed sufficient quantities of endogenous substrates to temporarily support stationary-phase transformation of CT even in the absence of a suitable, exogenous electron donor. At the concentrations used, there was 2 orders of magnitude less CT present in the reactors (on a mole basis) than there was O_2 in the 12-ml headspaces that contained air. Because CT was an insignificant drain on cellular reductant at the relative biomass and CT concentrations used, the benefit of adding a supplementary electron donor for CT biotransformation was probably realized only when the concentrations of inhibitory electron acceptors (e.g., molecular oxygen) exceeded the concentrations that could be consumed by the oxidation of endogenous substrates.

Electron acceptor experiments. CT dehalogenation by *S. putrefaciens* 200 did not occur in the presence of oxygen (data not shown). In microaerobically grown cultures containing 15 mM TMAO, fumarate, or Fe(III), CT was transformed at rates similar to the rates observed in cultures lacking any physiological electron acceptor (Fig. 1). However, transformation of CT in bottles containing nitrate was delayed by more than 100 h.

Since the typical rates of Fe(III) reduction by *S. putrefaciens* 200 (Table 1) would have resulted in depletion of Fe(III) within several hours, we performed additional experiments in which reduction of CT and reduction of Fe(III) were measured simultaneously. Our results showed that the rates of CT reduc-

TABLE 1. Rates of electron transfer to oxygen, nitrate, nitrite, and Fe(III) citrate by cultures of *S. putrefaciens* 200

Electron acceptor	No. of electrons transferred[a]	Rate of electron transfer by microaerobically grown cultures (nmol of electrons/min/mg of protein)[b]	Rate of electron transfer by aerobically grown cultures (nmol of electrons/min/mg of protein)[b]
Oxygen	4	4,300 ± 200	2,700 ± 200
Fe(III)	1	3,000 ± 1,500	570 ± 150
Nitrate	2	2,600 ± 700	3,000
Nitrite	3	2,100 ± 400	2,900
	2	1,400 ± 300	1,900
	1	700 ± 100	460

[a] The rates were determined by assuming that 4 mol of electrons was transferred for each 1 mol of O_2, 2 mol of electrons was transferred for each 1 mol of nitrate, and 1 mol of electrons was transferred for each 1 mol of Fe(III) reduced. Since the amounts of nitrite reduction products were not determined, the rates for various electron transfer stoichiometries for this compound are shown.

[b] Except for nitrate and nitrite reduction by aerobically grown cultures, the values are the means ± standard deviations of two to six measurements; for nitrate and nitrite reduction by aerobically grown cultures one measurement was obtained.

tion in bottles containing 50 mM Fe(III) citrate and bottles without Fe(III) citrate did not differ significantly (Fig. 2). The rates of Fe(III) reduction were almost constant over the first 1 h of the experiment; after 1 h more than 90% of the Fe(III) originally present was reduced to Fe(II). These results clearly showed that Fe(III) was present and did not reduce the rate of CT dehalogenation.

When 10 mM NO_3^- was added to microaerobically grown cultures, CT was not transformed until all of the nitrate and nitrite were consumed (Fig. 3). Although we performed duplicate experiments and obtained similar results, the results for only one replicate are shown in Fig. 3. In similar experiments in which 3 mM NO_2^- was used, CT transformation did not occur until the nitrite had disappeared (data not shown). A similar inhibition pattern [inhibition of dehalogenation by nitrate but not by Fe(III)] was observed with cultures grown under highly aerobic conditions (data not shown). Since NO_2^-

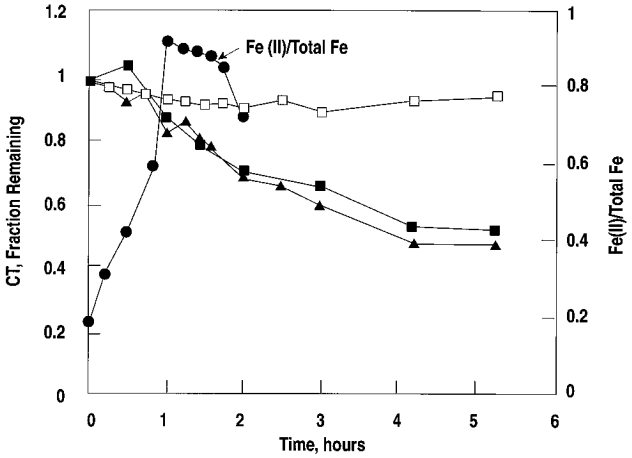

FIG. 2. Transformation of CT by microaerobically grown *S. putrefaciens* 200 in the presence of Fe(III). The initial concentration of Fe(III) citrate was 50 mM, the initial aqueous CT concentration was 3 ppm, and the optical density of the resuspended culture was 3.0. The values shown are the means of the values obtained for cultures in three replicate bottles. Symbols: ▲, CT in Fe(III)-containing bottles; ■, CT in bottles to which no Fe(III) citrate was added; □, CT in Westlake medium controls containing 50 mM ferric citrate; ●, ratio of Fe(II) to total Fe.

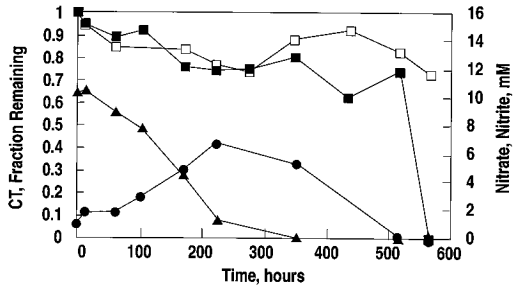

FIG. 3. Transformation of CT by microaerobically grown *S. putrefaciens* 200 in the presence of nitrate. The initial nitrate concentration was 10 mM. The initial culture optical density was 1.390. The initial mass of CT was 1.44 μmol (aqueous concentration, 3 ppm). Symbols: ■, CT in nitrate-containing bottles; □, CT in sterile water controls; ▲, nitrate; ●, nitrite.

is produced by NO_3^- reduction, the effects of nitrate on dehalogenation are not readily uncoupled from the effects of nitrite and/or other partial reduction products, and the identities of inhibitory nitrogen oxides cannot be determined unequivocally from the data.

In the presence of CT, reduction of NO_3^- occurred after a lag period of 1 to 2 days (Fig. 3), a lag period longer than expected for the induction of enzymes. Since a similar lag period was not observed in nitrate reduction experiments without CT, the lag period may have been a result of CT toxicity. On the basis of a protein concentration of 50 μg/ml for the cell suspensions used in the experiments shown in Fig. 3, the maximal nitrate reduction rate in the presence of CT was 27 nmol of electrons per min per mg of protein (assuming a two-electron reduction). This rate is approximately 1% of the rate of nitrate reduction observed in the absence of CT (Table 1).

Table 1 shows the results of electron transfer rate experiments performed with *S. putrefaciens* cultures grown under highly aerobic and microaerobic conditions. The O_2 and Fe(III) reduction rates were about 1.6 and 5.2 times greater, respectively, for microaerobically grown cultures than for cultures grown under highly aerobic conditions. Although the rates of reduction of all electron acceptors (Table 1) were higher than the rates obtained by DiChristina (9), in both studies there was a 1.5- to 2-fold increase in the amount of oxygen utilized following microaerobic growth. DiChristina observed increases in Fe(III) and nitrogen oxide reduction rates following microaerobic growth that were more pronounced than the increases observed in our study. Our rates of nitrate and nitrite utilization were not significantly affected by microaerobic growth. The reason for these differences is not known.

The effects of different electron acceptors on dehalogenation rates also provided insights into the arrangement of the electron transport chain of *S. putrefaciens*. Previously, Arnold et al. (3) suggested that inhibition of Fe(III) reduction by molecular oxygen resulted from the relative kinetics of electron transfer to Fe(III) and oxygen at branch points in the electron transport chain. To determine if a similar mechanism could explain the pattern of inhibition observed for CT dehalogenation, we determined the rates of electron transfer to nitrite, nitrate, oxygen, and Fe(III) citrate (Table 1). A satisfactory model of the physical arrangement and kinetic management of the electron transport chain in *S. putrefaciens* must be consistent with these respiration rate data and must explain (i) the complete inhibition of CT dehalogenation and Fe(III), NO_3^-, and NO_2^- reduction by O_2; (ii) the increased rates of CT dehalogenation and Fe(III) reduction following microaero-

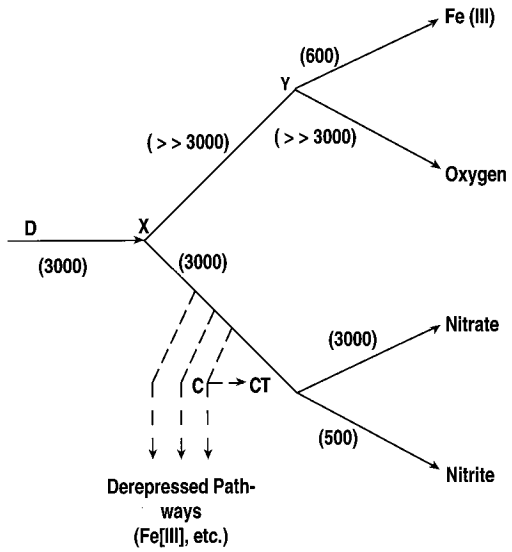

FIG. 4. Hypothetical branched electron transport chain of *S. putrefaciens* 200. Electrons enter the chain from the dehydrogenase (component D). The experimentally determined (see Table 1) maximal electron transfer rates (in nanomoles of electrons per minute per milligram of protein) are indicated along some branches and contribute to the proposed kinetic control mechanism. The solid lines indicate pathways that are present in cells grown under highly aerobic conditions. The dashed lines indicate components or branches that are derepressed during anaerobic or microaerobic growth.

bic growth; (iii) the complete inhibition of CT dehalogenation by NO_x but not by Fe(III); and (iv) the concomitant reduction of Fe(III) and NO_2^- or NO_3^- and the partial inhibition of Fe(III) reduction by NO_2^- or NO_3^- (9). These requirements are satisfied by the hypothetical electron transport chain shown in Fig. 4. In this proposed system, electrons from a dehydrogenase (component D) are directed toward an initial branch point, branch point X. In the simultaneous presence of O_2 and Fe(III), for example, the kinetics at branch point Y direct electrons to O_2. In the proposed scheme, branch point kinetics also ensure that essentially all of the electrons that reach branch point X are directed toward branch point Y in the simultaneous presence of O_2 and nitrate or nitrite. It follows that NO_x reduction is inhibited in the presence of O_2. In cells grown under aerobic conditions, electron transfer to oxygen is limited to a rate of approximately 3,000 nmol of electrons per min per mg of protein by metabolic events that precede branch point X.

We suggest that CT is dehalogenated at component C (which may represent a cytochrome) (18), which lies on one of several pathways that are partially repressed during growth under highly aerobic conditions. In our model, microaerobic growth results in greater production of depressed pathway components, including component C. Since anaerobic CT transformation occurs, albeit at a reduced rate, in cultures grown under highly aerobic conditions, aerobic repression of these components must be incomplete. Because the transfer of electrons to CT is relatively slow, we believe that component C must remain in its reduced form for dehalogenation to occur at all. Consequently, alternative pathways that can consume respiratory electrons at rates that are comparable to or greater than the maximal rates of substrate oxidation should impede dehalogenation reactions. Although the electron transport model shown in Fig. 4 explains most experimental observations concerning respiration by *S. putrefaciens* 200, it is no more than a working hypothesis at this point and should be refined as more information about the electron transport capabilities of the organism is obtained.

The mechanism by which NO_x inhibits Fe(III) reduction and CT transformation is not clear. Evidence obtained with another strain of *S. putrefaciens* (19) suggests that nitrate may be reduced to N_2O, N_2, or NH_4^+. Reduction to the ammonium ion would result in higher rates of electron transfer during NO_x reduction than those shown in Table 1 and Fig. 4 and would support a kinetic explanation for the observed patterns of NO_x inhibition. Inhibition by NO_x could also be a result of the production of NO as a product of NO_2^- reduction. NO is capable of binding to heme, iron-sulfur, and copper proteins to form metal-nitrosyl complexes (22) and of inhibiting the ability of the metalloprotein to transfer electrons (20). Since previous evidence has suggested that cytochrome *c* is involved in dehalogenation of CT by *S. putrefaciens* (18), inhibition of CT dehalogenation (and, possibly, inhibition of iron reduction) could be a result of formation of heme-nitrosyl complexes.

The results described above for *S. putrefaciens* 200 are particularly interesting when they are compared with the results obtained with *Pseudomonas* sp. strain KC (12, 21). Transformation of CT by strain KC occurs under denitrifying conditions but is inhibited by micromolar concentrations of ferrous or ferric iron. Since Fe-containing minerals are present at high concentrations in many sediments contaminated with haloorganic compounds, it is significant that CT dehalogenation in our experiments was not inhibited by Fe(III) or Fe(II). Although CT transformation by *S. putrefaciens* 200 is inhibited by nitrate and nitrite, such inhibition is transitory, and dehalogenation occurs at a high rate following NO_x reduction. The observed interspecies differences in dehalogenation ability suggest that the mechanisms by which and conditions under which haloorganic compounds can be transformed are diverse.

Petrovskis et al. recently described experiments similar to those described in this paper; in their study Petrovskis et al. used *S. putrefaciens* MR-1 (17). These investigators found that MR-1 also dehalogenates CT with production of chloroform and unidentified products. In contrast to our findings, however, Petrovskis et al. observed increased rates of CT transformation in the presence of Fe(III) citrate and did not observe inhibition by nitrate. These differences may have been due to metabolic variability between strains 200 and MR-1, but more likely resulted from interlaboratory variations in experimental procedures. Petrovskis et al. used (i) cultures that were two- to fivefold denser and (ii) electron acceptor concentrations that were 3- to 12-fold less than those used in our studies. Concurrent measurements of CT and electron acceptor contents during the course of their competition experiments were not reported by Petrovskis et al. It is possible that the electron acceptors which they used were depleted in a matter of minutes (i.e., prior to measurement of CT transformation).

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