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# Effects of electron acceptors and donors on transformation of tetrachloromethane by *Shewanella putrefaciens* MR-1

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**Abstract:** Transformation of chlorinated aliphatic compounds was examined in *Shewanella putrefaciens* strain MR-1, an obligately respiring facultative anaerobe. Under anaerobic conditions, MR-1 has been shown to transform tetrachloromethane to trichloromethane (24%), CO<sub>2</sub> (7%), cell-bound material (50%) and unidentified nonvolatile products (4%). The highest rate and extent of transformation were observed with MR-1 cells grown under iron(III)-respiring conditions. Lactate, formate and hydrogen were the most effective electron donors. Tetrachloromethane was not degraded in the presence of oxygen. Transformation of other chlorinated methanes and ethenes was not observed.

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**Key words:** *Shewanella putrefaciens*; Tetrachloromethane; Dechlorination; Biodegradation; Iron

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## Introduction

Reductive dehalogenation has been recognized as an important environmental transformation process of hazardous chlorinated solvents under anaerobic conditions. Microbial reductive dechlorination of alkyl halides has been observed using both axenic and mixed cultures [1,2]. Transformation of tetrachloromethane (CT) has been reported for axenic cultures under methanogenic,

acetogenic, and denitrifying conditions [3–7]. The mechanisms of chlorinated methane dehalogenation have been investigated using purified bacterial transition-metal coenzymes [8–11]. Whereas these systems may mimic in vivo dechlorination activity, the nature of the electron-transfer mediator has thus far not been conclusively determined [12].

*Shewanella putrefaciens* MR-1 is an obligatory respiring facultative anaerobe able to utilize Mn(IV) and Fe(III) oxides, as well as nitrate, trimethylamine N-oxide (TMAO) and fumarate as terminal electron acceptors [13–15]. Recently, *S. putrefaciens* strain 200 has been shown to transform CT, perhaps involving cytochrome *c* [16]. The effects of nitrate and Fe(III) on the

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transformation of CT by *S. putrefaciens* and by other bacteria are unclear. In some cases, nitrate [6,17] and Fe(III) [7] have been shown to inhibit the dechlorination of CT. The purpose of this study was to evaluate the ability of MR-1 to transform chlorinated aliphatic compounds and to evaluate the effects of electron acceptors and donors on the rates and extent of transformation of CT.

## Materials and Methods

### *Organisms and growth conditions*

MR-1 was isolated from anaerobic sediment of Oneida Lake, NY [13] and was grown in LM medium (100 mg l<sup>-1</sup> yeast extract, 100 mg l<sup>-1</sup> peptone, 50 mM NaHCO<sub>3</sub>, and 10 mM HEPES buffer, pH 7.5) containing 30 mM electron donor and 4–8 mM electron acceptor (iron(III) citrate, sodium nitrate, trimethylamine n-oxide (TMAO) or sodium fumarate). Medium was inoculated (1% v/v) with an aerobic late log-phase culture and incubated anaerobically in a 95% N<sub>2</sub>/5% H<sub>2</sub> atmosphere, within an anaerobic chamber. No chemical reducing agents were added. Cells were grown in 2-l Erlenmeyer flasks containing 1 l of medium at ambient temperature (approximately 22°C).

### *Dechlorination assays*

Dechlorination assays were performed in triplicate using resting cell suspensions. Cells were harvested at early stationary phase by centrifugation, washed three times and concentrated in a defined mineral medium [13] or in 10 mM HEPES buffer, pH 7.5, containing 100 mM electron donor and 4–8 mM electron acceptor, to achieve a total protein concentration of 0.3–1.0 mg ml<sup>-1</sup>. Final resuspension was done in an anaerobic chamber. For the electron donor study, cells were prepared in a N<sub>2</sub> atmosphere.

The dechlorination assay was performed with 1 ml of cells in 10 ml serum vials wrapped with aluminum foil and sealed with a Teflon-faced rubber septum and an aluminum crimp seal. Dechlorination was initiated upon addition of 0.01 μmol (20 μl) of chlorinated compound from

a sterile methanol or aqueous stock solution. The cells were incubated at 30°C. Dechlorination was monitored at regular time intervals, along with biotic (boiled or autoclaved cells) and abiotic (no inoculum) controls. Dechlorination assays were stopped by addition of 10 μl of 5 N NaOH, which raised the pH to higher than 10, thereby lysing the cells.

For determining a carbon mass balance, the dechlorination assay was done with [<sup>14</sup>C]CT (DuPont-NEN). Radiolabel present in cell-bound CO<sub>2</sub> and nonvolatile aqueous fractions was determined by a modification of a previously described method [18]. Triplicate vials containing 1.5 ml of cells were incubated at 22°C, rather than 30°C as above. Cell-bound labeled products were determined by pelleting the cells at 12000 rpm, washing, pelleting and resuspending in 10 mM phosphate buffer, pH 7.5. Cell suspensions were basified or acidified directly in the sealed assay vials and stripped for 15 min with N<sub>2</sub>. One ml of each fraction was added to 10 ml Ecolume (ICN) scintillation cocktail. For basified samples, 0.5 ml 1 N NaOH was added to the cocktail to keep the pH at 10. Identical cell suspensions were prepared with an equal mass of unlabeled CT to quantify CT and CF by gas chromatography.

### *Analytical procedures*

Samples were analyzed for chlorinated volatile compounds using headspace gas chromatographic analysis, according to a previously described method [10]. For chloride analysis, NADH (1 mM) was substituted as an electron donor, due to analytical interference by lactate. The assay was conducted in chloride-free 10 mM phosphate buffer, pH 7.5. Five 1.5 ml samples were transferred from headspace vials to microfuge tubes and centrifuged at 12000 rpm to pellet the cells. The supernatants were pooled, and chloride was analyzed on a Dionex Ion Chromatograph series 4500 i. Quantitation was based on triplicate injections on a Dionex Ionpac column (AS4A) with a Dionex Ionpac guard column (AG4A). Radiolabel was quantified with an LKB-Wallac 1219 liquid scintillation counter. Quenching was automatically corrected by the external standard channels ratio method. Total protein concentration was

determined by the Lowry assay [19], using bovine serum albumin as a standard. Cells were digested in 0.05 N NaOH at 60°C for 1 h before assay. Specific total cytochrome content was determined by the difference between the absorbances of the peak and trough of the Soret peak (420 nm) of a reduced-minus oxidized difference spectrum [20].

## Results and Discussion

### Screening of alkyl halide dechlorinating activity

Resting cell suspensions of anaerobically-grown *S. putrefaciens* MR-1 transformed tetrachloromethane (CT) to trichloromethane (CF) and unidentified products. CT was transformed by MR-1 cells after Fe(III)-respiring growth and resuspension in 10 mM HEPES buffer, pH 7.5 (Fig. 1A). Approximately 22% of the CT was dehalogenated to CF, and no further dechlorination products were observed. Abiotic and biotic controls (data not shown) did not show any CF production. With a Black Sea isolate [21]*S. putrefaciens*, strain MR-7, CT transformation yielded similar results to MR-1. Although no differences were observed in product distribution between the two strains, MR-7 exhibited faster specific rates of CT transformation (data not shown).

Dechlorination assays of [<sup>14</sup>C]CT were done with MR-1 to determine a carbon mass balance (Table 1). Assays were done in 10 mM HEPES buffer, pH 7.5. After Fe(III)-respiring growth, 49–58% of the transformed radiolabel was found to be cell-bound, 5–18% was recovered as CO<sub>2</sub> and 4% were unidentified aqueous intermediates. Chloride analysis indicates that the major transformation pathway involves the loss of a single chloride ion per molecule CT degraded (Table 1). After nitrate-respiring growth, a similar relative product distribution was obtained (Table 1). Mineralization of CT appears to be negligible, based on the statistically insignificant numbers obtained for [<sup>14</sup>C]CO<sub>2</sub> quantitation. The high fraction of cell-bound products suggests the occurrence of addition reactions by reactive intermediates, such as trichloromethyl radicals [6,16], at lipid double bonds [22]. The stoichiometry of CT transformation to CF by MR-1 corroborates recent results

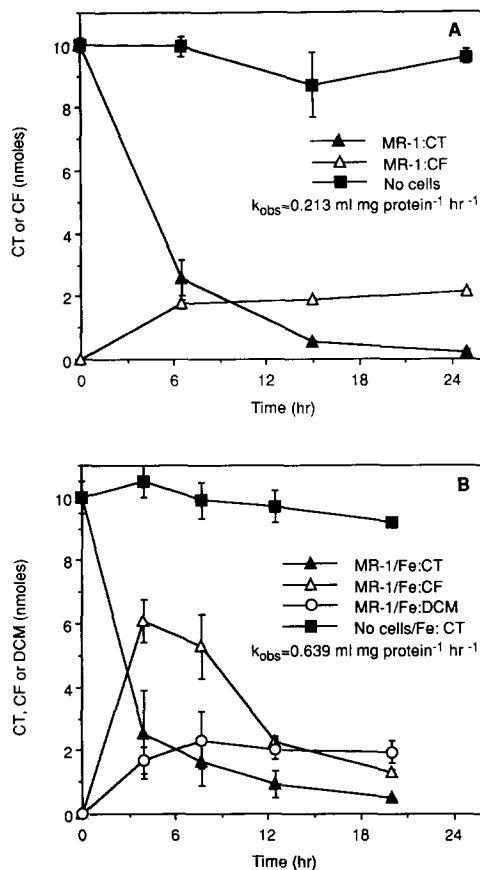


Fig. 1. Tetrachloromethane (CT) dechlorination and trichloromethane (CF) production by *S. putrefaciens* MR-1 resting cells after growth under Fe(III)-reducing conditions. CT transformation assays were done in the absence (A) and presence (B) of 8mM Fe(III) citrate. Total protein concentration was 0.83 mg ml<sup>-1</sup> (A) and 0.34 mg ml<sup>-1</sup> (B). Error bars represent  $\pm$  one standard deviation of triplicate samples.

obtained with *S. putrefaciens* strain 200 [16], although more CT was transformed and more cell-bound radiolabeled product was recovered in the current study. Other studies of CT transformation by anaerobic pure cultures have shown that reduction to CF and to dichloromethane (DCM) can occur in parallel to rapid transformation to CO<sub>2</sub>[3–7].

When lesser chlorinated products, such as trichloromethane (CF) and dichloromethane (DCM), were assayed with MR-1 and MR-7 resting cell suspensions harvested under nitrate- and fumarate-respiring conditions, no volatile dechloro-

Table 1

Stoichiometry of tetrachloromethane (CT) transformation by *S. putrefaciens* M/r-1 resting cells<sup>a</sup>

Electron acceptor	nmol CT added	nmol CT remaining	nmol CT lost <sup>b</sup>	Products						% total recovery <sup>d</sup>
				nmol CF	nmol cell-bound	nmol CO <sub>2</sub>	nmol aqueous intermediates	nmol chloride		
FE(III)	14.9 ± 0.5	1.04 ± 0.08	0.91 ± 0.34	2.92 ± 0.02	7.47 ± 0.44	0.71 ± 0.91	0.54 ± 0.04	13.0 ± 1.1	91	
	15.3 ± 0.1	0.89 ± 0.21	1.49 ± 0.95	2.41 ± 0.14	6.27 ± 0.10	2.35 ± 0.52	0.49 ± 0.02	ND <sup>c</sup>	91	
Nitrate	13.2 ± 0.5	3.97 ± 0.17	1.22 ± 0.54	2.12 ± 0.37	3.50 ± 0.06	0.34 ± 0.26	0.35 ± 0.06	9.3 ± 0.8	87	
	15.3 ± 1.1	3.78 ± 0.35	2.14 ± 0.70	2.73 ± 0.15	5.44 ± 0.26	0.27 ± 0.70	0.26 ± 0.02	ND <sup>c</sup>	96	

<sup>a</sup> Means ± one standard deviation of triplicate samples are shown. Incubation done at at 22°C for 45 h. When chloride was determined, the assays were done in 10 mM phosphate buffer, pH 7.5 and NADH (1 mM) was the electron donor. Otherwise, the assays were done in 10 mM HEPES, pH 7.5 and lactate (10 mM) was the electron donor.

<sup>b</sup> CT lost from abiotic controls.

<sup>c</sup> ND, not determined.

<sup>d</sup> Percentage of added CT recovered in unlabeled CT and CF and in [<sup>14</sup>C] products.

rination products were identified. Recoveries of CF and DCM were 92–95% after incubation times of up to 60 h. Similarly, no dechlorination products were identified when MR-1 or MR-7 cell suspensions were incubated with tetra- or trichloroethenes under nitrate- or fumarate-respiring conditions. This lack of activity may be due to structural or redox characteristics of the chlorinated compounds.

#### Effect of anaerobic electron acceptor conditions

The effects of different electron acceptors on transformation of CT were examined by assaying dechlorinating activity of MR-1 resting cell suspensions, previously harvested under various electron acceptor growth conditions. Lactate and

hydrogen were the electron donors in all cases. CT transformation assays were done with and without electron acceptor present. For cells grown under nitrate-, TMAO- or fumarate-respiring conditions, the presence of electron acceptor in the CT assay had no effect on the CT transformation rate (data not shown). However, for cells grown under Fe(III)-respiring conditions, the addition of 8 mM Fe(III) as iron citrate to the dechlorination assay resulted in a three-fold increase in specific CT transformation rate (Fig. 1B). DCM was also produced, presumably due to abiotic reduction by Fe(II), as CF was not otherwise degraded by MR-1. CF was transformed to DCM in MR-1 cell suspensions incubated with Fe(III) (data not shown). No CF or DCM were

Table 2

Effect of electron acceptors on transformation of tetrachloromethane (CT) by *S. putrefaciens* MR-1 resting cells<sup>a,b</sup>

Electron acceptor <sup>c</sup>	CT added (nmol)	CT remaining (nmol)	CF produced (nmol)	% Recovery <sup>d</sup>	k <sub>obs</sub> (ml mg protein <sup>-1</sup> hr <sup>-1</sup> )	Specific cytochrome content <sup>e</sup>
FE(III)	10.1 ± 0.32	0.20 ± 0.09	2.16 ± 0.03	23	0.177 ± 0.057	1.04 ± 0.17
Nitrate	11.2 ± 0.03	1.54 ± 0.09	1.54 ± 0.05	28	0.105 ± 0.039	0.66 ± 0.12
TMAO	9.40 ± 0.28	4.54 ± 0.59	0.66 ± 0.16	55	0.053 ± 0.018	0.99 ± 0.01
Fumarate	10.9 ± 0.07	5.04 ± 0.45	0.91 ± 0.08	60	0.029 ± 0.004	1.01 ± 0.19

<sup>a</sup> Means ± one standard deviation of triplicate samples of a representative experiment are shown. Observed first-order rate constants (k<sub>obs</sub>) were determined for three independent experiments. Cytochrome content was determined for duplicate independent experiments.

<sup>b</sup> Incubation times were 15 h for nitrate- and 24 h for Fe(III), TMAO- and fumarate-respiring conditions.

<sup>c</sup> Electron acceptors used for growth and present in CT transformation assays. For cells harvested after growth under Fe(III) respiring conditions, no Fe(III) was present in the CT transformation assays.

<sup>d</sup> Total recovery of CT and CF.

<sup>e</sup> The difference between the peak and trough of the Soret peak per mg of total protein.

observed in abiotic controls which also contained 8 mM Fe(III). Reductive dechlorination of CT has been previously observed in solutions containing Fe(II) [23].

The relative rates of CT transformation were highest for Fe(III)-respiring cells, followed by nitrate-, TMAO- and fumarate-respiring cells (Table 2). The extent of CT transformation was related to the electron acceptor used for growth. Cell suspensions prepared after growth under Fe(III) ( $E^\circ = +770$  mV), nitrate ( $E^\circ = +430$  mV), TMAO ( $E^\circ = +130$  mV) or fumarate ( $E^\circ = +30$  mV) reduction demonstrated CT transformation rates proportional to these  $E^\circ$  potentials. With less energy available from carbon oxidation using terminal electron acceptors with more negative oxidation-reduction potentials, *S. putrefaciens* may have fewer electrons available for the reduction of CT. These results contrast with observations made with *E. coli*, where a greater fraction of CF was produced under fermentative conditions, when compared to fumarate-respiring conditions [6]. CT transformation rates did not show correlation to specific total cytochrome content for any of the anaerobic growth conditions examined. The specific total cytochrome content for nitrate-respiring cells was approximately 67% of that for Fe(III)-, TMAO- or fumarate-respiring cells (Table 2). With *S. putrefaciens* 200, CT transformation rates increased with increases in specific cytochrome *c* content [16].

#### Effect of oxygen on reductive transformation

Anaerobically (Fig. 2A) or aerobically (Fig. 2B) grown MR-1 cell suspensions containing atmospheric oxygen during the CT transformation assay were unable to significantly dechlorinate CT. No measurable CF production was detected. Atmospheric oxygen is known to inhibit reduction of anaerobic electron acceptors in MR-1 [13]. Although oxygen concentrations were not determined, these results suggest that oxygen also prevents CT transformation. When assayed under oxygen-free conditions, even aerobically grown MR-1 cells dechlorinated CT to CF (Fig. 2B), although to a lesser extent than anaerobically grown cells (Fig. 2A). Addition of chlorampheni-

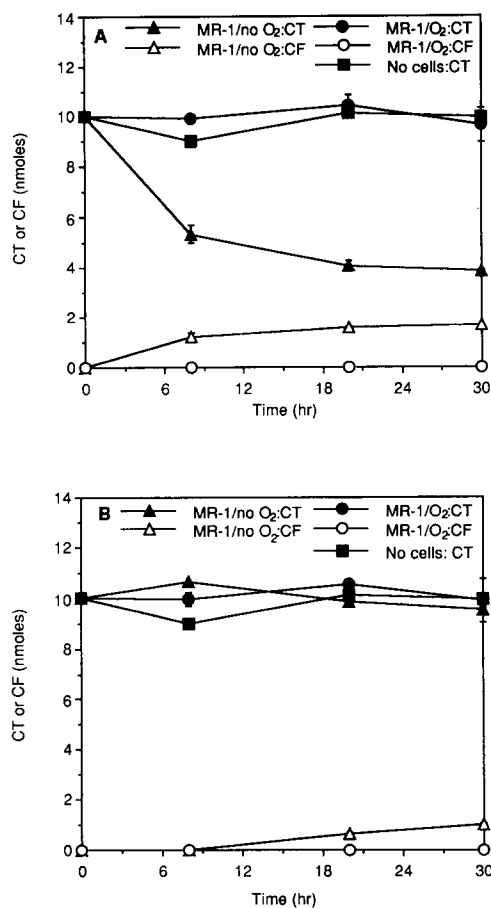


Fig. 2. Tetrachloromethane (CT) dechlorination by *S. putrefaciens* MR-1 resting cells after nitrate-respiring (A) or aerobic (B) growth. Assays were done in the absence (▲, △) or presence (●, ○) of oxygen. Total protein concentration was 0.80 mg ml<sup>-1</sup>. Error bars represent ± one standard deviation of triplicate samples.

col to these assays further inhibited this transformation by 94% after 49 h (data not shown). Aerobically grown MR-1 is reported to be unable to translocate protons in response to anaerobic electron acceptors [15]. Furthermore, MR-1 increases the synthesis of cytochromes [24] and quinones [25] several-fold during anaerobic growth. Thus, induction of synthesis of anaerobic respiratory components may be necessary for significant CT transformation activity.

### Effect of electron donors

The effect of different electron donors on CT dechlorination was determined using resting cell suspensions harvested after growth on lactate under nitrate-respiring conditions with lactate. CT transformation rates were enhanced 2.5–3.0-fold when lactate, formate, hydrogen or acetate and succinate, were added to resting cell suspensions. Significant additional CF production relative to unamended controls was not observed when methanol was added. Lactate was expected to be the most effective electron donor, since the cells were grown with lactate as a carbon and energy source in a 5% H<sub>2</sub> atmosphere and since lactate oxidation to acetate yields more electrons than formate oxidation to CO<sub>2</sub> or than H<sub>2</sub> oxidation. However, these effects may not be measurable, since the concentration of electron donor in the dechlorination assay is in excess of CT concentration.

In summary, *S. putrefaciens* MR-1 and MR-7 nonstoichiometrically dechlorinated CT to CF under oxygen-free conditions. Approximately 50% of radiolabeled CT was transformed to cell-bound material (which was not sorbed based on chloride release) with lesser amounts as CO<sub>2</sub> and non-volatile aqueous intermediates. The rate and extent of transformation were greatest with cells grown under Fe(III)-respiring conditions. No inhibition of CT transformation was observed in the presence of nitrate, TMAO or fumarate. However, oxygen inhibited CT transformation. Furthermore, anaerobic growth was required for significant CT transformation activity. Increased CT transformation rates were observed with the addition of lactate, formate or hydrogen as electron donors. Preliminary results indicated that in iron(III)-reducing environments, bacteria may transform chlorinated pollutants directly, as well as generate a reductant, Fe(II), for abiotic dechlorination.

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