Rapid Dechlorination of Carbon Tetrachloride and Chloroform by Extracellular Agents in Cultures of *Methanosarcina thermophila*

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Previous experiments in our laboratory suggested the existence of an excreted biomolecule from *Methanosarcina thermophila,* capable of rapid carbon tetrachloride (CT) and chloroform (CF) transformation. Batch experiments with filtered supernatants from *M. thermophila* cultured in the presence and absence of elemental iron (Fe⁰) were performed to determine if such a molecule existed. Filtering the supernatant with a 0.22 μm filter did not alter the CT or CF transformation rate or pattern. Supernatants from cultures grown in medium without Fe⁰ were able to facilitate rapid CT transformation, but could not catalyze subsequent CF transformation. However, when the organisms were cultured in the presence of high concentrations of Fe⁰ (10 g/L), the filtered supernatant catalyzed rapid CT and CF transformation. Autoclaving the supernatant did not significantly alter the CT transformation rate or pattern; however, heat treatment decreased CF transformation. The extracellular factor appeared to have a high transformation capacity for CT; upon dechlorinating 82 μm CT, the rate of transformation did not decrease.

Introduction

Halogenated aliphatics comprise the largest class on the list of priority pollutants compiled by the US Environmental Protection Agency (*1*). Many of these compounds are known or suspected human carcinogens or mutagens. Carbon tetrachloride (CCl_4, CT) is a widespread groundwater contaminant (*2*), with concentrations ranging from several parts per billion (*2,3*) to the parts per million level (*4*). Chloroform ($CHCl_3$, CF) is the first hydrogenolysis product of CT. Chlorinated solvents such as CT and CF can be resistant to degradation and can therefore persist in the environment.

Previous research in our laboratory suggested the existence of one or more excreted factors (referred to as EF) from the methanogen *Methanosarcina thermophila,* which catalyzed rapid CT and CF degradation (*5*). The EF was present when the organisms were cultured in growth medium; however, when the organisms were cultured in the presence of elemental iron (Fe⁰), the culture supernatant demonstrated enhanced CT and CF transformation ability.

Organisms can respond to external stimuli by excreting biological molecules. Kim et al. (*6*) found that when a copper-resistant methanogen, *Methanobacterium bryantii* BKYH, was exposed to high concentrations of copper, the organism responded by excreting 4-fold increased levels of three extracellular proteins. These proteins were thought to protect the organism from toxicity. Extracellular factors that are gratuitously active in the transformation of contaminants can also exist. Dybas and co-workers (*7*) have found a *Pseudomonas* species that excretes a small biomolecule that transforms CT to CO_2 under denitrifying conditions. *M. thermophila* may respond to Fe⁰ by excreting a biomolecule, also serendipitously active in the dechlorination of CT and CF.

Materials and Methods

Chemicals. All chemicals were high-pressure liquid chromatography (HPLC) grade. Unwashed Fe⁰ powder supplied by Aldrich Chemical Co. was used in all of the experiments (99.9% pure, 10 μm, specific surface area of 2.02 m²/g). Methane, H_2–CO_2 (80:20 vol/vol), and N_2–CO_2 (80:20 vol/vol), were obtained from Air Products.

Organism and Culture Conditions. *M. thermophila* (DSM 1825; Deutsche Sammlung von Mikroorganismen, Braunschweig, Germany) was cultured as described. The medium contained (per liter of NanoPure water (Barnstead)), 0.0048 g of $NiCl \cdot 6H_2O$, 0.010 g of $Fe(NH_4)_2 \cdot (SO_4)_2 \cdot 6H_2O$, 2.50 g of Na_2CO_3, 1.26 g of NH_4Cl, 0.5 g of yeast extract, 0.5 g of tryptic soy broth, 1.13 g of K_2HPO_4, 0.905 g of KH_2PO_4, and 0.001 g of resazurin. Ten milliliters per liter of a vitamin solution (*8*), 10 mL/L Wolfe's minerals (*8*), and 37.7 mL/L mineral II solution (per liter of NanoPure water: 6.0 g of KH_2PO_4, 4.8 g of NH_4Cl, 12.0 g of NaCl, 2.4 g of $MgSO_4 \cdot 7H_2O$, 1.6 g of $CaCl_2 2H_2O$) were added. The medium was decanted into anaerobic 140-mL bottles (Supelco) with thick butyl-rubber stoppers (Bellco), or into a 1-L Pyrex bottle fit with a constructed stopper (*5*). Prior to autoclaving, Fe⁰ powder (1 g/100 mL of media) was added to half of the bottles containing medium. Bottles were sealed, autoclaved, and cooled.

Experimental Procedure. Crimp-top serum bottles (140 mL), with and without Fe⁰, were inoculated with *M. thermophila* (5% inocula, by volume). Once the cultures had reached the stationary phase, as monitored via methane concentration, the bottles were uncapped in a glovebag (Coy), decanted into centrifuge tubes (25 mL each), sealed, and centrifuged at 5700g for 15 min. The supernatants were decanted directly into sterile 38-mL bottles in the glovebag. No Fe⁰ or cells were present in the supernatant systems used in this research. Supernatants decanted from organisms cultured in the presence and absence of Fe⁰ are referred to as "+Fe" and "–Fe", respectively. Medium controls consisted of 25 mL of sterile growth medium with a N_2/CO_2 headspace. All experiments were performed with triplicate bottles for each treatment. Bottles were incubated at 50 °C quiescently on their sides for the duration of each experiment.

Stoppers were made to minimize loss of CT and CF via volatilization or sorption. Thick butyl rubber stoppers (Bellco), with the bottom 2 mm removed, were affixed to the bottom 2 mm of a Teflon-covered rubber stopper (Supelco) with "Plumber's Goop" adhesive (Eclectic Products, Inc.) so that the bottle contents were exposed to Teflon. These stoppers were used in all experiments.

Aqueous stock saturated solutions of CT and CF were made with approximately 3 mL of neat CT or CF in 22 mL of NanoPure water. The solutions were shaken on a wrist action shaker for several hours and allowed to settle at 20 °C for several days before use. Stock calibration standards were

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prepared gravimetrically in methanol with neat CT and CF. Calibration standards were prepared from stock standards in 38-mL bottles containing 25 mL of NanoPure water.

Each experiment was started with the addition of 5−10 μm CT (total concentration) to the bottles. Headspace samples were taken for GC analysis, and CT and CF were monitored with time.

CT and CF Degradation by Culture Supernatants. The +Fe and −Fe supernatant systems described above were prepared. In addition, one set of +Fe and −Fe supernatants were filtered through disposable 0.22 μm sterile syringe filters (Millipore). These treatments are referred to as +Fe filtered supernatant and −Fe filtered supernatant, respectively. One set of medium controls was also prepared.

Effect of Autoclave Treatment on CT and CF Degradation by Culture Supernatants. Serum bottles were prepared as described above with +Fe and −Fe supernatants filtered with 0.45 μm syringe filters (Millipore). Two sets of +Fe and −Fe supernatant, and one set of medium controls were prepared. One set each of the −Fe and +Fe supernatant bottles was autoclaved for 10 min at 118 °C and allowed to cool to room temperature. The autoclaved bottles are referred to as −Fe autoclaved and +Fe autoclaved for those containing −Fe and +Fe supernatant, respectively.

Effect of Size Fractionation on CT and CF Degradation by Culture Supernatants. Serum bottles were prepared as described above with +Fe and −Fe supernatants filtered with 0.45 μm syringe filters; however, only 15-mL samples were used. The +Fe and −Fe supernatants were spun (45 min, 1000g) through a centrifuge−filter unit (Centriplus Concentrators, Amicon, Inc.) while N_2 was blown into the centrifuge unit. This allowed a filtrate of fluid and molecules <10 kDa through the filter, but retained molecules >10 kDa on the filter. After 45 min the concentrators were placed back in the anaerobic glovebag. Once returned to an anaerobic environment, the samples were reduced back to the methanogenic redox level (as determined by the color change in resazurin without the addition of more reductant). The filtrate (<10 kDa) from each of the concentrator tubes was placed into sterile 38-mL bottles, which were then sealed. The retentate (>10 kDa) from each concentrator tube was resuspended in 15 mL autoclaved medium, placed into sterile 38-mL serum bottles, and sealed. One set of medium controls was also prepared.

Determination of the Culture Supernatant Transformation Capacity. Sets of +Fe supernatant, −Fe supernatant, and medium control bottles were prepared and filtered with a 0.45 μm syringe filter as described above. CT was added to each bottle (5−13 μM) the bottles were shaken, and 15−30 min later, a headspace sample for GC analysis was taken. Samples were periodically taken throughout a 24-h period, and when the CT had been transformed either completely or almost completely (approximately every 24 h), more CT was added to the bottles. An additional spike of CT was added to the controls on day 5 of the 12-d experiment. A total of 9 CT spikes were added to the supernatant systems.

Data Analysis. Since the concentration of the EF was unknown, and because the different treatments were handled in as similar a manner as possible, a first-order rate coefficient calculation was thought to provide the most suitable means by which comparisons between treatments could be made. Rate coefficients were calculated as follows. Degradation of CT (C_C) is represented by:

$$\frac{dC_C}{dt} = -K'C_C$$

Where K' is the first-order rate coefficient and t is time. This expression can be integrated, yielding the equation:

TABLE 1. First-Order Rate Coefficients for CT Degradation by Supernatant from *M. thermophila* Cultures[a]

treatment	CT (d^{-1})
+Fe not filtered	15.3 ± 0.14
+Fe filtered	15.6 ± 0.48
−Fe not filtered	5.71 ± 0.58
−Fe filtered	5.96 ± 0.30
medium-only	0.52 ± 0.08

[a] Note: Centrifugation was used to provide the supernatant.
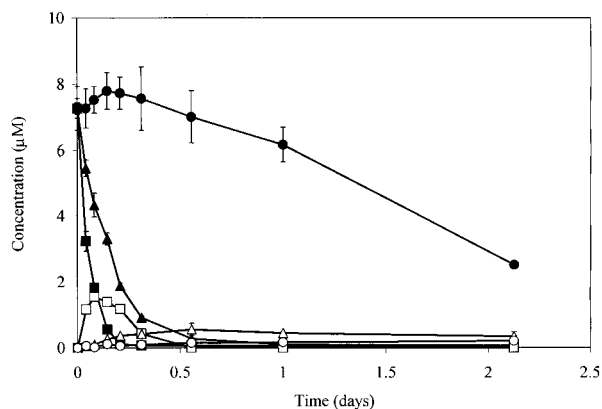

FIGURE 1. Degradation of CT and formation and subsequent degradation of CF by supernatants of *M. thermophila* cultures. Error bars represent ± one standard deviation. Symbols are as follows: ■, CT, +Fe-filtered supernatant; □, CF, +Fe-filtered supernatant; ▲, CT, −Fe-filtered supernatant; △, CF, −Fe-filtered supernatant; ●, CT, medium-only; ○, CF, medium-only treatments. The unfiltered supernatant treatments have been deleted for clarity. The profiles from these treatments lay directly on top of the filtered supernatant treatments.

$$-\ln\frac{C_C}{C_{C0}} = K'(t - t_0)$$

where C_{C0} is the initial CT concentration at time zero (t_0). When $-\ln(C_C/C_{C0})$ is plotted against time, t, the slope is equal to K'. Two-sided paired t-tests over the first-order transformation coefficients were performed between various treatments at the 95% confidence level (*9*).

CT and CF Measurement. Headspace samples (100-μL were taken with a gas-tight locking syringe (Precision Sampling Corp., Baton Rouge, LA) and injected into a gas chromatograph (Hewlett-Packard 5890 series II) equipped with an electron capture detector (GC-ECD) for CT and CF measurement. A fused silica capillary column (J & W) with a DB-5 stationary phase was used. Nitrogen was the carrier and makeup gas. The oven temperature was held at 35 °C for a run length of 2.5 min. Method detection limits were 0.00034 μM and 0.025 μM for CT and CF, respectively (method 1030 E, *10*).

Results and Discussion

CT and CF Degradation by Culture Supernatants. The supernatant component previously proven to contribute to CT degradation (*5*) was not removed by filtration through a 0.22 μm filter (Table 1). This indicates that the active supernatant component is most likely an excreted biomolecule rather than cells or Fe^0 particles. First-order transformation coefficients for this experiment are listed in Table 1. Transformation of CT and subsequent formation and transformation of CF are shown in Figure 1. There was no statistically significant difference between the CT transformation coefficients in the filtered and unfiltered systems,

TABLE 2. First-Order Rate Coefficients for CT Degradation (d^{-1}) in Culture Supernatants Which Have or Have Not Been Autoclaved

treatment	CT
−Fe not autoclaved	12.3 ± 1.98
−Fe autoclaved	10.0 ± 0.26
+Fe not autoclaved	16.7 ± 0.95
+Fe autoclaved	15.4 ± 1.46
medium-only	0.53 ± 0.12

FIGURE 2. CF formation and subsequent transformation by autoclaved supernatants of *M. thermophila* cultures. Error bars represent ± one standard deviation. Symbols are as follows: ■, +Fe supernatant not autoclaved; ○, +Fe supernatant autoclaved; △, −Fe supernatant not autoclaved; ▲, −Fe supernatant autoclaved; ●, medium-only treatments.

nor did filtration change the CT and CF transformation patterns in these systems. However, the +Fe supernatant and −Fe supernatant CT transformation coefficients were significantly different from each other. The +Fe supernatant (filtered and nonfiltered) degraded CT and CF much faster than all other systems; both +Fe and −Fe supernatant systems degraded CT and CF faster than medium controls (Table 1, Figure 1).

Medium controls showed some loss of CT over the course of all of the experiments; however, this loss was minimal in the time frame of the experiment when compared to the other systems (Figure 1, Table 1). All systems were incubated at 50 °C, which was thought to increase the volatilization rate of CT. Some CT was dechlorinated to CF (Figure 1); this was most likely due to nucleophilic attack from bisulfide in the medium (*11*). No loss of CF was seen in medium controls in any of the experiments.

The enhanced transformation was not thought to be due to the carryover of microscopic and colloidal Fe^0 particles. Although a 0.22 μm filter may not remove all traces of microscopic and colloidal Fe^0, if Fe^0 catalyzed this transformation, filtering should change the CT and CF transformation rates and patterns; this was not observed. In addition, when in an abiotic system, Fe^0 does not rapidly catalyze the transformation of CF (*5, 12*). Previously determined Fe^0-catalyzed transformation coefficients for CF were 0.31 ± 0.03 day^{-1} and 0.50 d^{-1} (refs *5* and *12*, respectively). The +Fe supernatants (and the suspected EF) were very effective in promoting CF transformation; thereby suggesting that colloidal Fe^0 did not catalyze this transformation. Furthermore, enhanced transformation of CT was also seen in the −Fe supernatant treatments, which would contain no Fe^0.

It is hypothesized that a biological EF was excreted when *M. thermophila* was in the presence of Fe^0, and this EF was active in the transformation of CT and CF. Because the bottles from the −Fe systems degraded CT and CF significantly faster than the medium control, it was also suggested that the organisms alone may secrete some type of EF that enhanced degradation. When cells were cultured in the presence of Fe^0 it is possible that (1) an additional EF was excreted, (2) a higher concentration of the EF present in the −Fe supernatant system was excreted, or (3) the EF present in the −Fe supernatant system was further reduced, thereby increasing its activity. The differences in CF formation and transformation profiles (Figure 1) suggested that the +Fe supernatant system did not contain a higher concentration of the EF present in the −Fe supernatant system.

Effect of Autoclaving on CT and CF Degradation by Culture Supernatants. The effect of autoclaving the culture supernatant was explored to determine the heat sensitivity of the EF. First-order transformation coefficients for CT with or without autoclave treatment are listed in Table 2. Formation (from fed CT) and subsequent transformation profiles of CF for this experiment are given in Figure 2. Autoclaving did not significantly reduce the CT transformation rates in the −Fe and +Fe systems. However, autoclave treatment did decrease the ability of the EF to transform CF (Figure 2). CF transformation occurred readily only in the
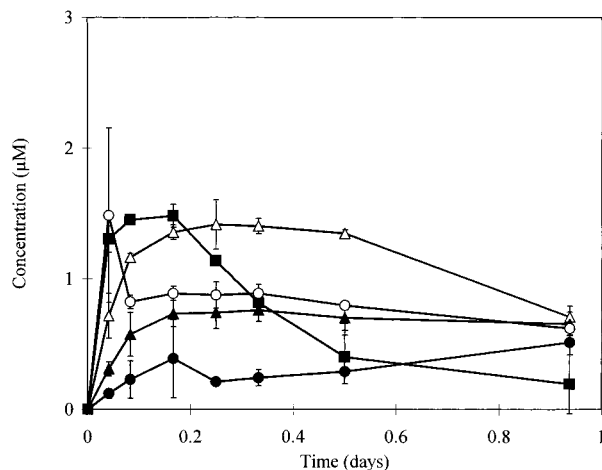
not-autoclaved +Fe supernatant. In the −Fe not-autoclaved supernatant, slow CF transformation occurred. In all autoclaved supernatants no CF transformation took place.

Porphyrins containing redox-active metals, thought to mediate dechlorination reactions, remain stable upon autoclave treatment (cofactor B_{12} for example, ref *13*). However, proteins, whether alone or surrounding a metal, typically do not. Experimental evidence suggests that the EF responsible for the transformation of CT could contain a metal center, while the catalyst involved in CF transformation contains a protein component. Molecules composed of a protein containing a metal center are common; examples include cytochrome *c*, cofactor B_{12}, and cofactor F_{430} (*14*). The protein enhances the overall activity of the molecule, perhaps stabilizing the complex or reducing interference with other molecules, yet the actual dechlorination takes place at the redox metal center. Because CT is easier to dechlorinate than CF, less of an effect should be observed in the rate of CT versus CF transformation upon autoclaving if the EF were such a molecule. It is also possible that two different factors catalyze the transformation of CT and CF and the CF-transforming EF is heat labile, whereas the CT-transforming EF is not.

Effect of Size Fractionation on CT and CF Degradation by Culture Supernatants. In initial experiments with the concentrator tubes, the samples were exposed to oxygen for ~8 h. All systems demonstrated very limited activity (data not shown). It was thus concluded that oxygen destroyed extracellular CT and CF degradation abilities and precautions were taken to limit oxygen exposure in additional experiments.

Because samples required centrifugation for filtration in the concentrator tubes, some oxygen exposure was necessary. However, the length of oxygen exposure was limited to ~30−45 min and was reduced by blowing N_2−argon gas into the centrifuge unit. Samples were immediately returned to the glovebag after centrifugation.

CT transformation is shown in Figure 3. There was an initial lag period in the samples prior to the transformation of CT; in the calculation of rate coefficients for CT transformation, only those data points after the lag period were taken into account. CT transformation rate coefficients were not significantly different in the +Fe retentate and filtrate, and the −Fe retentate and filtrate samples. All of the supernatant treatments degraded CT at a significantly faster rate than the medium control.
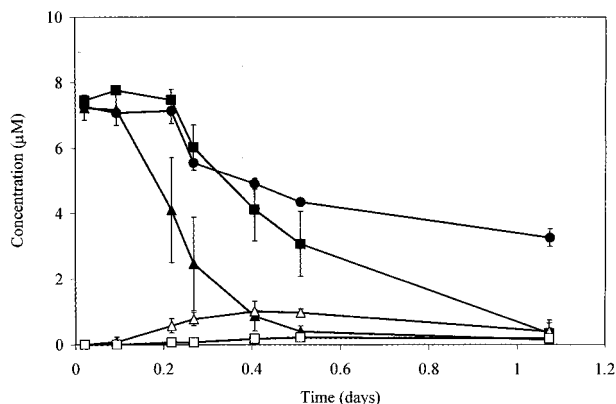
FIGURE 3. Degradation of CT and formation and subsequent degradation of CF by *M. thermophila* culture supernatants that have undergone size fractionation. Error bars represent ± one standard deviation. Symbols are as follows: ▲, CT, +Fe supernatant retentate; △, CF, +Fe supernatant retentate; ■, CT, +Fe supernatant filtrate; □, CF, +Fe supernatant filtrate; ●, CT, medium-only treatments. The −Fe supernatant treatments have been deleted for clarity.
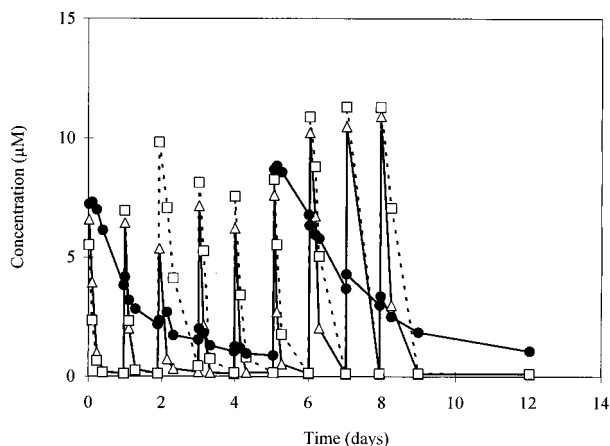

FIGURE 4. Degradation of CT in the transformation capacity experiment. Symbols are as follows: ●, medium-only; △, +Fe supernatant; □, −Fe supernatant treatments. Error bars are omitted for clarity.

The results for CF transformation showed that the degradation activity was in the retentate fraction (>10 kDa). Very little CF was formed in the filtrate treatments (both the −Fe and +Fe systems), and no subsequent CF degradation was observed. In the −Fe retentate system, about 7% of the initially fed CT was dechlorinated to CF, and this CF was slowly degraded. Approximately 13% of the initially fed CT was degraded to CF in the +Fe retentate treatment, and the CF was more rapidly transformed.

Because transformation rate differences in the retentate and filtrate fractions were not statistically different, the size of the EF was not definitively determined. It is possible that the EF contains active components in both the retentate and the filtrate fractions. The fraction >10 kDa would include proteins and molecules larger than proteins, and the fraction <10 kDa could include porphyrins which are typically <2 kDa in size (*13,14*).

Determination of the Culture Supernatant Transformation Capacity. An experiment to explore the transformation capacity of the culture supernatant was performed to determine the dechlorination limit of the EF. CT and CF concentration profiles are shown in Figures 4 and 5, respectively. CT was added to the +Fe and −Fe treatments on days 0, 1, 2, 3, 4, 5, 6, and 8. CT was added to the medium control on day 0 and 5.
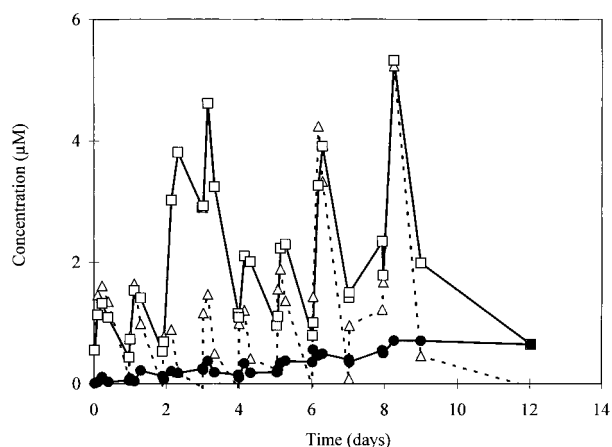

FIGURE 5. Formation and degradation of CF in the transformation capacity experiment. Symbols are as follows: ●, medium-only; △, +Fe supernatant; □, −Fe supernatant treatments. Error bars are omitted for clarity.

CT and CF were transformed much faster in the +Fe system than in the −Fe system; however, the CT transformation capacity appeared to be approximately the same in both systems and the transformation limit was never approached in this experiment. Slow removal of CT did occur in the medium control, for reasons discussed above. CF appeared to build up to some extent in the −Fe supernatant system, while it was immediately transformed in the treatment containing +Fe supernatant. The difference in the ability of CF to be readily transformed in the presence of supernatants from the +Fe system versus the −Fe system has been observed consistently in all of the experiments conducted with the *M. thermophila* supernatants. It appears that when sufficient time is given between spikes, CF can be completely transformed in the −Fe treatment; however, when spikes occur in quick succession, as between days 2 and 5, CF is likely to build up.

Evidence for electron-donor depletion or inactivation of the EF (*15−17*) was not observed in these systems. Both treatments dechlorinated approximately 82 μM CT (about 2.1 μmol CT/bottle) in 12 d. During the experiment there was no decrease in the rate of CT transformation. There was an excess of electron donor present for reducing CT or CF, possibly via the rereduction of the EF. Because the bottles were filled in the glovebag, there was a small quantity of H_2 gas in the headspace of each bottle; therefore, it is possible that H_2 may serve as an electron donor in EF-catalyzed dechlorination.

The nature of the extracellular factor or factors is, as of yet, uncertain. The molecule(s) could be a porphyrin of some type, or a metalloprotein or proteins with no relationship to a porphyrin. There is also the possibility that an aggregate of extracellular material, polysaccharides, or inactive proteins for example, has bound or complexed with Fe^0 and has thereby become catalytically active. The enhanced extracellular dechlorination of CT and CF is almost certainly biological in origin and as more work is performed to isolate this factor, more will be known about its potential uses and benefits.

Acknowledgments

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