Novel Approaches for Bioremediation of Organic Pollution

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PREFACE

Pollution of the environment with organic compounds, mostly due to industrial activities, has become a major environmental issue. The situation is getting worse due to the continued release of large amounts of chemicals and the broadening of the spectrum of xenobiotics that to which the biosphere is exposed to.

The field of biodegradation and bioremediation has experienced a dynamic and intense period of development during the last few years, encompassing all its different disciplines: Isolation and characterization of new microorganisms with novel catabolic activities, understanding the specific enzymatic mechanisms underlying biodegradation, rational design of genetically engineered degrading microorganisms, and studying the interaction of the degrading organism with biotic and abiotic components within the environment to allow development of efficient bioremediation processes.

This book, devoted to the proceedings of the 42nd OHOLO Conference on "Novel Approaches for Bioremediation of Organic Pollution" which was held in Eilat, Israel, in May 1998, obviously does not cover the full range of bioremediation research. However, an attempt has been made to address the major issues in bioremediation of organic pollution. The four sessions of the conference covered a wide spectrum of topics, from the lab research— characterization of enzymes and pathways, genetic design of degradative microorganisms, to the field application issues—bioaccessibility, bioavailability, process development and field studies.

The contributions aimed at understanding of enzymes and pathways are those on the transformation of halogenated and sulfur containing compounds, the elucidation of different pathways for degradation of nitroaromatic compounds, and the necessity of considering streochemistry in biodegradation reactions.

The genetic approaches for the design of engineered degradative microorganisms are exemplified by presentations on the generation and use of biocatalysis database on the World Wide Web, the manipulation of the catalytic performance of dehalogenases and esterases either by the well established strategy of rational site-directed mutagenesis or by the recently developed "irrational" approach of directed evolution. Other genetic approaches presented are using controlled *gfp* expression as a reporter for sensing bioavailability and the construction of a contained engineered degrading strain by the application of regulated suicide systems.

Several strategies for *in situ* bioremediation application are discussed, including specialized techniques of bioaugmentation and bioavailability enhancement using biosurfactant-producing strains, a combined active/passive biotreatment strategy, monitoring

biological degradation of organic pollution by determination of $^{13}C/^{12}C$ isotopic fractionation, and use of engineered *lux* gene fusion based biosensor strains for both bioremediation and process monitoring. Recent developments in *ex situ* bioremediation processes, like a new membrane reactor concept, are presented as well.

Although these proceedings can not cover all the recent advances in the field of biodegradation and bioremediation of organic pollution, we believe that the various strategies presented here reflect the major trends of research and development for the coming years.

We would like to take this opportunity to thank again all the contributors to this book, and to the members of the scientific advisory committee: Kenneth Timmis, Gary Sayler, Eugene Rosenberg, Yigal Henis, Gabi Amitai, Baruch Velan, and Avigdor Shafferman.

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CHEMICAL STUDIES OF CARBON TETRACHLORIDE TRANSFORMATION BY *Pseudomonas stutzeri* STRAIN KC

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1. INTRODUCTION

Carbon tetrachloride (CT) is a toxic, carcinogenic compound which was widely used in the past as a solvent, degreaser, fumigant, and fire extinguisher. As a result of earlier disposal practices, CT is now found in groundwater. Probably the most extensive example of groundwater contamination can be found at the Hanford Nuclear Reservation operated by the U.S. Department of Energy in south-central Washington state (Illman, 1993). An estimated 1000 metric tons of CT was disposed of into unlined cribs over a 20-year period. CT has percolated through the vadose zone, contaminating groundwater over 250 feet below the surface. The plume of contamination extends over approximately 7 square miles and is migrating toward the Columbia River, only a few miles away.

CT is inherently stable in aqueous solution with a slow rate of spontaneous hydrolysis (Jeffers *et al.*, 1996). Biological destruction of this chemical offers the potential of a low-cost technology to eliminate its risk if a suitably effective transformation activity can be identified and promoted *in situ*. Since the carbon atom of CT is at its highest formal oxidation state, the molecule offers no potential nutritional advantage to microbes as a source of energy. It can serve as an oxidant of suitable biological redox carriers, and several studies have demonstrated reductive dehalogenation using biochemical cofactors (Gantzer and Wackett, 1991; Krone *et al.*, 1989a,b; Wackett *et al.*, 1992). These transformations have primarily involved hydrogenolysis, yielding the net replacement of chlorine atoms with hydrogen to form chloroform (CF) and the lesser chlorinated chloromethanes.

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These methods have the potential for creating new hazards with longer half-lives under the reducing conditions necessary for their formation from CT. Bacterial cultures have been screened for their ability to transform CT co-metabolically, that is, while using other compounds supplied as sources of carbon and energy and with no known selective advantage for carrying out CT transformation. These examples of cometabolism have involved enrichments or pure cultures grown under anaerobic conditions to promote reduction of the CT without the competition assumed to result from molecular oxygen (Bouwer and McCarty, 1983; Criddle et al, 1990a,b; Egli *et al.*, 1988; Mikesell and Boyd, 1990). Some of these studies have identified pure cultures of microorganisms capable of carrying out a net hydrolysis of CT to give CO_2 as a major product without the accumulation of CF or other chloromethanes (Criddle *et al.*, 1990a; Egli et al., 1988). One such culture which we found particularly attractive for our study was a strain of the denitrifying bacterium *Pseudomonas stutzeri* designated KC (Criddle *et al.*, 1990a). As a facultative anaerobe isolated from an aquifer, this organism had characteristics which seemed optimal for a site such as the Hanford Reservation.

In order to assess whether the CT transformation activity of strain KC is suitable for the design of remediation technology, and to allow the rational design of such a technology, a detailed knowledge of the process is necessary. Previous physiological characterizations had shown that the activity was present only in cultures grown under conditions of iron deprivation (Criddle *et al.*, 1990a; Lewis and Crawford, 1993; Tatara *et al.*, 1993) that there was a requirement for trace levels of copper (Tatara *et al.*, 1993), that growing cells were required (Dybas *et al.*, 1995), and that the process was inhibited by oxygen but was still observed under aerobic conditions (Lewis and Crawford, 1993). More recently it has been shown that the factor or factors elaborated by strain KC that allow CT transformation were extracellular and of low molecular weight (<500 daltons; Dybas *et al.*, 1995), and were active with several types of microbial cultures, but not as extracellular preparations alone (Tatara *et al.*, 1995). Chemical characterization of the activity has included gas chromatographic analyses of CT transformation products, which have shown CF to be a minor product (Lewis and Crawford, 1993), and tracer studies using ^{14}CT, which identified CO_2 (approximately 55%) and an uncharacterized nonvolatile fraction (approx. 40%) as the major products (Criddle *et al.*, 1990a).

Two pathways to explain the net hydrolysis of CT have been proposed, both of them including an initial reduction step. The first pathway proposed to explain CO_2 production from CT involved a two-electron reduction to give dichlorocarbene, which would hydrolyze to give formate and/or CO (Figure 1; Criddle *et al.*, 1990a). CO_2 could then be generated from these compounds by oxidation with an appropriate dehydrogenase, provided that one was present and had sufficiently low K_m values to efficiently oxidize the low concentrations of substrate formed from the micromolar quantities of CT used in these studies.

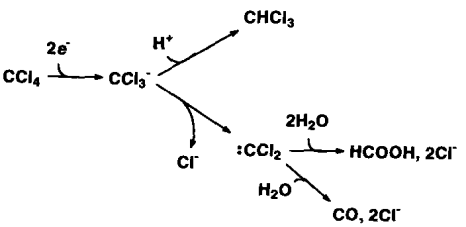

Figure 1. Two-electron reduction of CT.

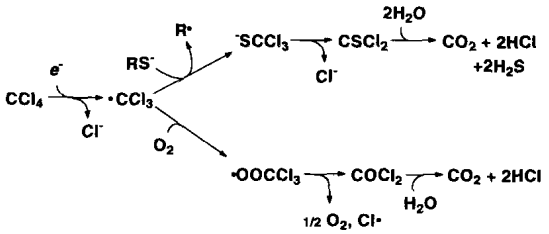

Figure 2. One electron reduction/radical substitution pathway of CT transformation.

The second pathway involves a one-electron reduction to give trichloromethyl radical (Figure 2). Trichloromethyl radical is known to react with molecular oxygen at near the diffusion-limited rate (Slater, 1980) and to give phosgene (Asmus *et al.*, 1985), a reactive electrophile which hydrolyzes to CO_2. Another fate of trichloromethyl radical has been proposed to explain the formation of carbon disulfide and CO_2 from CT in mineral systems under anaerobic conditions (Kriegman-King and Reinhard, 1992). This reaction involves combination of the radical with a reactive sulfur species to give thiophosgene, which can undergo further substitution with sulfur or hydrolyze to give CO_2.

Both of these pathways include reactive intermediates which would not accumulate to measurable quantities in our experiments due to their brief half-lives in aqueous media. We have tried to resolve the relative contribution of each of these pathways to the spectrum of products seen in cultures of strain KC using trapping agents designed to give stable products diagnostic of the respective intermediate, and quantitative product analyses.

2. MATERIALS AND METHODS

2.1. Culture Conditions

Cultures were grown on Medium D (Criddle *et al.*, 1990a), or a medium based on it, but which obviated the need for precipitation of metals. This medium, designated DRM, consisted of 6g K_2HPO_4, 2g sodium acetate, 1g NH_4Cl, and 0.5g $NaNO_3$ per liter, adjusted to pH 7.7–7.9 and autoclaved. $MgSO_4$ (1 mM), $Ca(NO_3)_2$ (0.1 mM), and $CuCl_2$ (5 nM) were added after autoclaving from autoclaved stock solutions. Cultures used to study CT transformation were inoculated from aerobically grown overnight cultures in the respective medium. CT transformation was conducted under anaerobic conditions by transferring 5-ml cultures in 10-ml serum vials to an anaerobic chamber (Forma Scientific, atmosphere: 90%N_2, 10%H_2) before adding CT and sealing with sterile, Teflon-faced butyl rubber stoppers (the West Co., Phoenixville, PA). CT was added from a 10 mg/ml methanolic stock solution. Cultures were incubated at 25°C for 4 days in an inverted position. When O_2 was included it was provided by flushing the headspace with breathing grade O_2 which was passed through a sterile, 0.45-μ (pore size) filter before closing with a stopper. CT was then added by quickly removing and replacing the stopper.

2.2. Analytical Methods

CT and CF were quantitated as described by Lewis and Crawford (1993). Carbon disulfide was quantitated using a Hewlett Packard 5890 gas chromatograph and G1909

purge and trap concentrator with a 5-ml sample vessel, and interfaced with a 5989 mass spectrometer operated in single ion mode (m/z = 76 a.m.u., Hewlett Packard, Avondale, PA). An 11-min purge was used to transfer organic analytes to a trap composed of Tenax, silica gel, and activated charcoal (Supelco, Inc., Bellefonte, PA). Helium was used as the carrier gas. The column used was a 25 m × 0.32 mm PoraPLOT Q (Chrompack, Middelburg, The Netherlands) held at 40°C for 4 min, and then increased to 220°C at 30°/min and held for 5 min. Calibration was performed using a set of external standards spanning the range of concentrations encountered in culture samples.

Fractionation of radioactivity was performed by purging acidified cultures through a trapping train using N_2 at a flow rate of 100 ml/min. The purged vapors passed through Teflon and stainless steel tubing and into three vials of 10-ml Ready Organic scintillation cocktail (Beckman Instruments, Fullerton, CA) sealed with Viton gaskets, and one vial with 10 ml of Carbosorb CO_2-trapping solution (Packard Instruments, Meriden, CT) and a butyl rubber gasket. Vials of Ready Organic were counted directly and 1 ml of the Carbosorb was mixed with 10 ml EcoLite scintillation cocktail (Research Products International, Mount Prospect, IL) before counting in a Packard 2100TR liquid scintillation analyzer. Radioactivity trapped in Ready Organic was designated as "volatile" products; that trapped in Carbosorb as "CO_2"; and that remaining in the medium as "nonvolatile" products. HPLC separation of nonvolatile products was performed using a Hewlett Packard 1090 HPLC system with a diode array detector monitoring at 210 nm. Conditions for separation of products from trapping experiments included a 4.6 × 250 mm Microsorb MV C_{18} column (Rainin Instruments Inc, Emeryville, CA), and acetonitrile (A) and 15 mM H_3PO_4 0.1% triethylamine (B) as eluting solvents at a flow rate of 1 ml/min. The solvent gradient used was 99% B, 0–3 min; 50% B, 16–21 min; 30% B, 23–35 min; 99% B, 37 min. [^{14}C]Formate was analyzed using the same HPLC system but with a Rezex ROA column (Phenomenex, Torrance, CA, 7.8 × 300 mm with 50-mm guard column) operated at 40°C with 5 mM H_2SO_4, 0.5 ml/min, as eluent. Radioactivity in HPLC effluent was quantitated using a β-RAM liquid scintillation detector (IN/US Systems, Inc., Tampa, FL) and IN-Flow scintillation cocktail at 3 ml/min. A calibration curve relating peak area to disintegrations per minute was made using [^{14}C]formate.

2.3. Chemicals

CT and CF of Omnisolv grade were obtained from E. Merck and Co. (Cherryhill, NJ); [^{14}C]CT (4.3 mCi/mmol) from DuPont NEN (Boston, MA); [^{14}C]CS_2 (57 mCi/mmol) from Amersham International (Buckinghamshire, UK); 2,2,6,6-tetramethylpiperidinyl-1-oxyl (TEMPO), hydroxyurea, and sodium [^{14}C]formate from Sigma (St. Louis, MO); and [^{13}C]CT from Cambridge Isotope Laboratories (Andover, MA).

3. CHEMICAL STUDIES OF REACTIVE INTERMEDIATES OF CT TRANSFORMATION BY STRAIN KC

Previous studies have shown that products indicative of thiophosgene and phosgene could be identified using nucleophilic trapping agents such as N,N′-dimethylethylenediamine (DMED; Lewis and Crawford, 1995). Quantitative analyses of this type of experiment and one including the dichlorocarbene-trapping agent 2,3-dimethylbutene (DMB) are shown in Figure 3. DMED substantially diminished production of CO_2 from CT, whereas DMB had no significant effect on this product fraction. The nonvolatile frac-

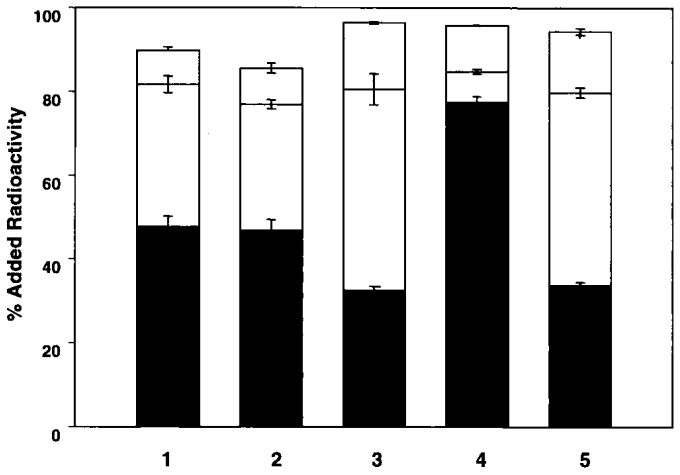

Figure 3. Product profiles of ^{14}CT transformation by strain KC in the presence of trapping agents. Bottom (black) bars represent non-volatile products, middle (white) bars represent CO_2, top (gray) bars represent volatile material trapped in organic scintillation cocktail. 1, strain KC control (no trapping agents); 2, 5 mM DMB; 3, 5 mM formate; 4, 5 mM DMED; 5, strain KC control. Experiments 1 and 2 were done in parallel with Medium D; experiments 3, 4, and 5 were done in parallel with DRM. Results are means of triplicate experiments with error bars representing ± one standard deviation.

tion from experiments including DMED were analyzed to determine if the products seen were those expected of thiophosgene and phosgene trapping. Reversed phase HPLC and liquid scintillation detection showed that the major products eluted were the sulfur- and oxygen-substituted imidazole products seen previously, 2-thioxo-1,3-dimethylimidazole (TDMI) and 1,3-dimethylimidazolidinone (DMIO), respectively. Those products accounted for approximately 66% of the eluted radioactivity (>90% recovery of injected radioactivity). The remainder of the radioactivity eluted in an unknown peak or rather broadly over the radiochromatogram in an anaerobic experiment (Figure 4A). When oxygen was included in the headspace, a profound increase in the oxygen-substituted product was seen, along with a complementary decrease in the amount of the sulfur-substituted product and the unknown peak (Figure 4B).

Though sulfur and oxygen-substituted products clearly could account for the majority of CT transformation products in the trapping experiments mentioned above, it was possible that carbon disulfide was included in the pathway leading to these products. CS_2 can hydrolyze to give CO_2 and can react directly with DMED to give one of the observed products (TDMI). In addition, an intermediate of CS_2 hydrolysis, carbonyl sulfide, can react with DMED to give both of the observed products. CS_2 could be detected by purge and trap GC/MS in cultures of strain KC which had transformed CT. Using ^{13}CT as a mass tracer confirmed that this was a product of CT transformation and not of normal metabolism in the medium used (Figure 5).

Quantitation of CS_2 by this method indicated that it accounted for only 1.8 ± 0.6% of the total CT removal. It was possible that CS_2 accumulated to low steady-state levels due to its further metabolism by the culture. To assess the reactivity of CS_2 in this system, $^{14}CS_2$ was added in an amount similar to CT additions used previously (235 nmol/10-ml vial). In these experiments no apparent CS_2 hydrolysis activity was demonstrated by strain KC when compared with sterile controls. The amount of radioactivity found in base traps used to collect CO_2 from cultures or controls was less than 1.5% of the total. The nonvola-

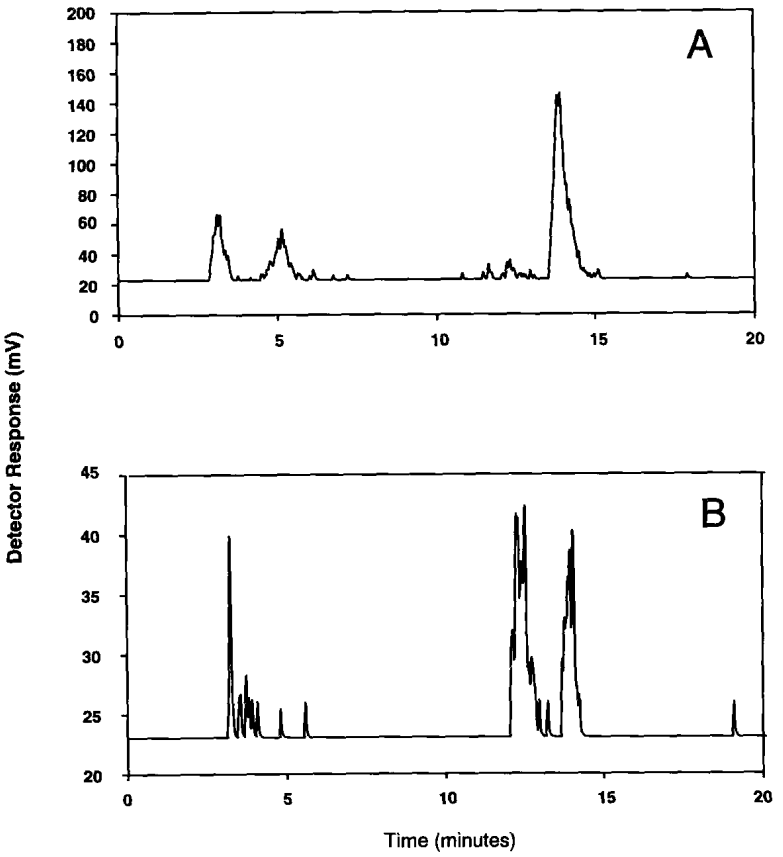

Figure 4. HPLC analysis of DMED-trapped, nonvolatile CT transformation products. A, data from sample of anaerobic culture containing 5mM DMED. B, data from sample of culture incubated with an O_2 headspace and 5mM DMED.

tile products formed in the presence of DMED, TDMI and an unidentified polar fraction, were the same in sterile controls and KC-inoculated cultures, though their relative quantities were reversed (mostly polar material with KC). No DMIO was detected in these experiments.

Other products of CT transformation were examined since their routes of formation and quantities would contribute to a more complete description of the CT-transformation process elicited by strain KC. Chloroform may result from trichloromethyl radical abstracting a hydrogen atom from an organic molecule in the surrounding medium. As mentioned, CF accumulation accounts for a small portion of the total mass balance for CT; however, some phenomena became apparent during our studies which may allow further insight into participating reactive intermediates. We had observed CF accumulations of up to 3% of the CT transformation when a medium buffered with 50 mM N-2-hydroxyethylpiperazine-N'-ethanesulfonic acid (HEPES) was used. This CF production was lower when oxygen was present in the culture vessels (Lewis and Crawford, 1993). Previously, Criddle et al. (1990a) had observed no CF when a phosphate-buffered medium was used. In further experiments with a phosphate-buffered medium, we were able to observe detectable levels of CF which were much lower than with the HEPES-buffered medium. This suggested that the organic buffer had participated in the radical-scavenging

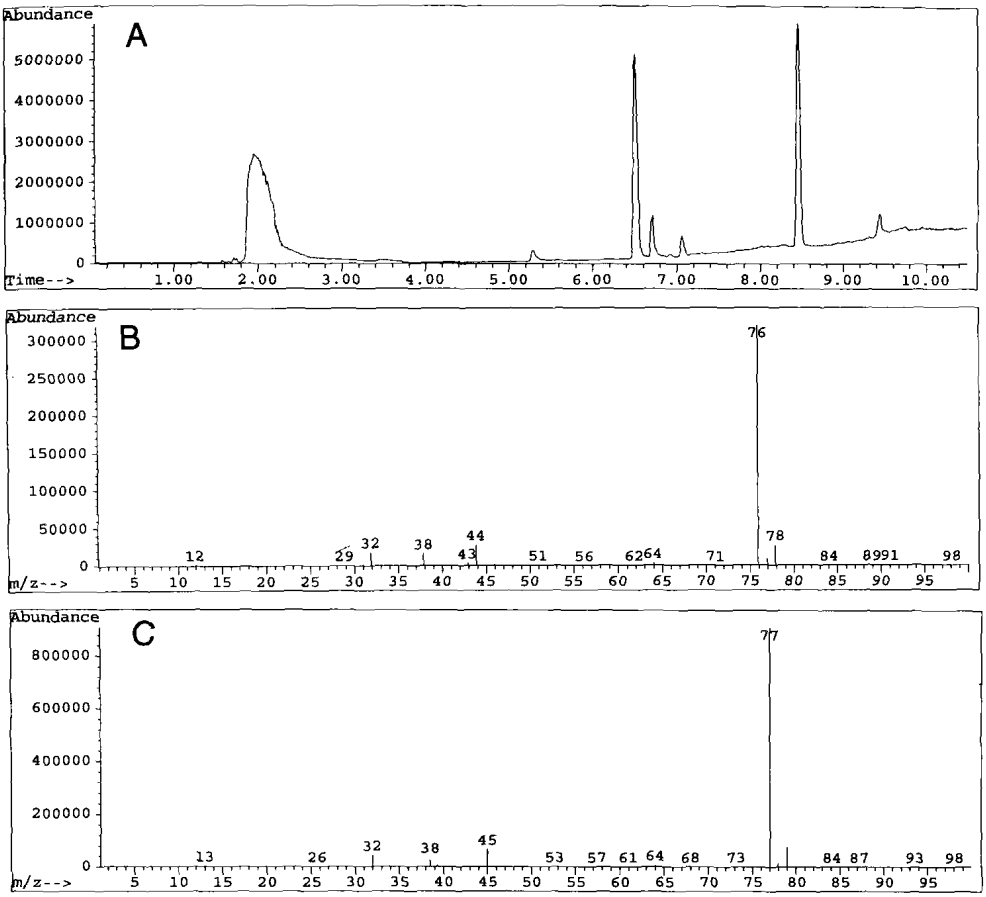

Figure 5. *GC/MS identification of carbon disulfide as a product of CT transformation by strain KC. A. GC/MS chromatogram (ions m/z 21–300 a.m.u.) of volatiles trapped by purge and trap concentration from 5 ml of strain KC culture exposed to CT. B. Mass spectrum of CS_2 peak eluting at 7.07 min in above chromatogram. C. Mass spectrum of CS_2 peak from culture grown with ^{13}CT. Peaks eluting earlier than CS_2 include CO_2, 2.0 min; ethanol, 5.27 min; acetone, 6.52 min; and isopropanol, 6.71 min. These peaks were not labeled by ^{13}C. Peaks eluting later than CS_2 include CF, 8.45 min; and CT, 9.42 min. These were present initially.*

reaction leading to CF. We conducted another series of experiments to test whether a known radical scavenger, hydroxyurea, and a stable free radical, TEMPO, would influence the accumulation of CF in a manner consistent with their predicted reactivity with trichloromethyl radical. Hydroxyurea is an effective inhibitor of ribonucleotide reductase of some organisms by virtue of its radical-quenching effect (Lammers and Follman, 1983). However, *P. stutzeri* has been shown to possess a corrinoid-type of ribonucleotide reductase (Gleason and Hogenkamp, 1972), and strain KC is resistant to high concentrations of this compound (our unpublished results). Addition of 50 mM hydroxyurea resulted in a ninefold increase in CF accumulation (Table 1). The addition TEMPO resulted in a lower mean CF accumulation in this and other experiments ($P \geq 0.1$).

Formate has been identified as a product of CT transformation by strain KC (Dybas *et al.*, 1995). In our system, when [^{14}C]formate was quantitated from tracer experiments using organic acid HPLC and liquid scintillation detection, radioactivity eluting in the re-

Table 1. Effect of radical scavengers on CF formation

Addition	Mole % CF [a]
None	0.33 ± 0.03
50 mM hydroxyurea	2.73 ± 0.27
1.3 mM TEMPO	0.20 ± 0.05

[a] means ± standard deviation of triplicate cultures analyzed in duplicate

gion corresponding to formate accounted for approximately 4.5% ± 0.6% of the total CT addition. To determine if this amount was the net result of formate production and formate oxidation, 5 mM formate was added in an experiment to examine the relative contribution of formate and formate dehydrogenase to $^{14}CO_2$ production. This addition of formate at more than 300-fold molar excess to the CT addition had no significant effect on CO_2 production (Figure 3, 48 ± 3.7% with formate versus 46 ± 1.2% without formate, ≥96% recovery of ^{14}C), as had been noted previously (Dybas *et al.*, 1995).

4. DEVELOPMENT OF A CELL-FREE ASSAY OF CT TRANSFORMATION ACTIVITY

Studies of cometabolic transformation of xenobiotic chemicals using bacterial cultures are complicated by the fact that they represent the process being studied as well as metabolic functions which support that activity. Consequently, in studies designed to determine the type(s) of factors elicited by strain KC, the effects of inhibitors on the process under study cannot be resolved from their possible effects on cell metabolism. In addition, determining the stoichiometry of electrons consumed per CT molecule transformed would be very difficult with growing cultures. For these purposes, a highly defined cell-free system is necessary. Tatara and co-workers (Dybas *et al.*, 1995; Tatara *et al.*, 1995) have described a system whereby CT transformation activity could be observed in strain KC culture supernatants. Those experiments used cells of several types of bacterial cultures and a yeast, presumably as a source of reductant; the supernatant alone had little or no activity. They also determined that this activity would pass through a low-molecular-weight-cutoff membrane. We have studied this activity first by testing chemical reductants rather than whole cells. In these experiments, a crude cell-free supernatant was prepared by centrifugation and filtration through a 0.2-μ (pore size) membrane. Without added reducing agent, no CT removal was seen; however, with either Ti^{III} citrate or dithiothreitol significant CT removal was seen in overnight incubations. As a test of whether the addition of these potent reductants gave an artifactual CT removal not related to the activity seen with whole cells, these reactions were conducted with supernatants prepared from cultures known to be inactive for CT transformation, and with substrates toward which strain KC has shown no activity. Some of these results are shown in Figure 6.

Only iron-limited KC culture supernatant showed CT removal in this system. In addition, no activity was seen toward CF or fluorotrichloromethane ($CFCl_3$), consistent with the activity described for whole cultures (Criddle, 1990b; Lewis and Crawford, 1993) and not with that of reduced cofactors such as vitamin B_{12} (Gantzer and Wackett, 1991; Krone et al, 1989b). The addition of iron or iron-supplemented culture supernatant did not inhibit the activity of the iron-limited culture supernatant, indicating that this system is not sensitive to any "exacerbating factors" observed in studies with whole cells (Dybas et al., 1995). This activity is not sensitive to 2 mM sodium azide, which inhibits the growth of