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Physiology of Carbon Tetrachloride Transformation by Pseudomonas stutzeri KC

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PHY

PHYSIOLOGY OF CARBON TETRACHLORIDE TRANSFORMATION BY *PSEUDOMONAS STUTZERI* KC

By

Gregory Michael Tatara

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ABSTRACT

PHYSIOLOGY OF CARBON TETRACHLORIDE TRANSFORMATION BY *PSEUDOMONAS STUTZERI* KC

By

Gregory M. Tatara

Pseudomonas stutzeri KC transforms carbon tetrachloride (CCl_4) under denitrifying conditions without the production of chloroform. Trace metal studies established that iron played an inhibitory role in the transformation, leading to the development of a hypothesis which stated an iron scavenging agent is responsible for the cometabolic transformation of CCl_4. Localization experiments established that both a small (< 500 dalton) secreted factor and cells of *P. stutzeri* KC were required to transform CCl_4. Initial purification experiments determined that the secreted factor was extractable with acetone, and further purification of this factor revealed that activity was recoverable following semi-preparative HPLC analysis. Based on published studies for iron scavenging systems, it was hypothesized that a diverse range of other microbial species and genera can be used in combination with the secreted factor to achieve CCl_4 transformation. This hypothesis was proven true and lead to the development a bioassay for the secreted factor using *Pseudomonas fluorescens*. The bioassay established that in addition to *P. stutzeri* KC cells grown under denitrifying conditions, aerobically-grown strain KC produces the factor, however oxygen reversibly inhibits CCl_4 transformation. The bioassay was also used to establish that live cells are needed for CCl_4 transformation, and the factor is stable indefinitely after lyophilization to powder. Preparation of crude cell membranes demonstrated that membranes in combination with the secreted factor rapidly transformed CCl_4 when NADH was added as a source of reducing equivalents. Inhibitor studies established that the respiratory electron-transport chain was not involved

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in CCl_4 transformation. *Lactobacillus acidophilus*, an organism lacking any membrane-associated electron-transfer-proteins, did not result in CCl_4 transformation when combined with the secreted factor. These studies lead to the development of a new model which posits that CCl_4 accepts an electron(s) from a reduced form of the secreted factor produced during growth in medium that contains copper and is iron limited. The oxidized form of the secreted factor is then capable of further transformations only after reduction at the cell-membrane by a non-respiratory enzyme .

I humbly dedicate this dissertation to my loving parents

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CHAPTER 1

INTRODUCTION:
TRANSFORMATIONS OF CARBON TETRACHLORIDE

Halogenated aliphatic compounds that contaminate groundwater pose a hazard to human health, and thus their degradation to innocuous products by biotic and abiotic systems is of interest. Carbon tetrachloride (CCl_4) degradation is of particular interest because of its toxicity and carcinogenicity. CCl_4 adversely affects the eyes, liver, kidneys, central nervous system, and skin of humans. Excessive exposure can result in central nervous system depression and gastrointestinitis, while acute exposure can result in the condition of toxic hepatitis [48].

The non-polar and nonflammable character of CCl_4 made this molecule a very useful solvent. CCl_4 has been used in the manufacture of fire extinguishers, refrigerators, aerosols, and chlorinating organic compounds including chlorofluoromethanes [51]. It has also been used as a degreasing agent for metals, an agricultural fumigant, an extractant, and a solvent for oils, fats, lacquers, varnishes, rubber, waxes, and resins [48, 51]. An estimated 5 million lbs/year of CCl_4 were emitted during manufacture and processing, and approximately 60 million lbs/year were released as solvent emissions [48]. Because of its widespread use and improper disposal, CCl_4 has become a significant pollutant of soil and groundwater. In 1985, CCl_4 was reported in 10% of 113 public water supplies surveyed, at mean concentrations of 2.4 to 6.4 μg/L, and in 25% of groundwater supplies at concentrations of 1 to 400 μg/L [48]. Estimates indicate that 19 million people are exposed to CCl_4 through ambient air, 20 million through contaminated drinking water, and 2 million through contaminated soils and landfills at levels greater than the EPA exposure standard of 5 μg/L. [48]. Thus, the environmental remediation of CCl_4 contamination is of scientific and practical significance.

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Mechanisms and pathways of CCl_4 transformation

Known pathways for CCl_4 are shown in Figure 1.1. Although CCl_4 may theoretically undergo direct hydrolysis, it is generally believed that the first step of this mechanism is the one-electron reduction of CCl_4 to give a trichloromethyl radical and a chloride ion (1) [13]. Depending on the environmental or experimental conditions, the trichloromethyl radical can undergo one of several possible reactions: dimerization of the trichloromethyl radical to give hexachloroethane (2), interaction with a lipid to form chloroform(3); sequential reduction first to chloroform and subsequently, in sufficiently reduced environments to dichloromethane (4), a second one-electron reduction to form a dichlorocarbene radical which can undergo hydrolysis to carbon monoxide(CO) and formate (5); reaction with HS- to form thiophosgene which can subsequently react to form CO_2 (6), addition of molecular oxygen to form phosgene which can hydrolyze to CO_2 (7); and lastly, covalent bonding to cell material (8). Typically, several pathways operate simultaneously and competitively in both biotic and abiotic systems. The numbers shown next to the pathways in Figure 1.1 correspond to numbering of the text in the following sections. The text provides documentation and a discussion of each pathway, followed by a section discussing the relationship between the known CCl_4 transformation pathways and transformation by *P. stutzeri* KC.

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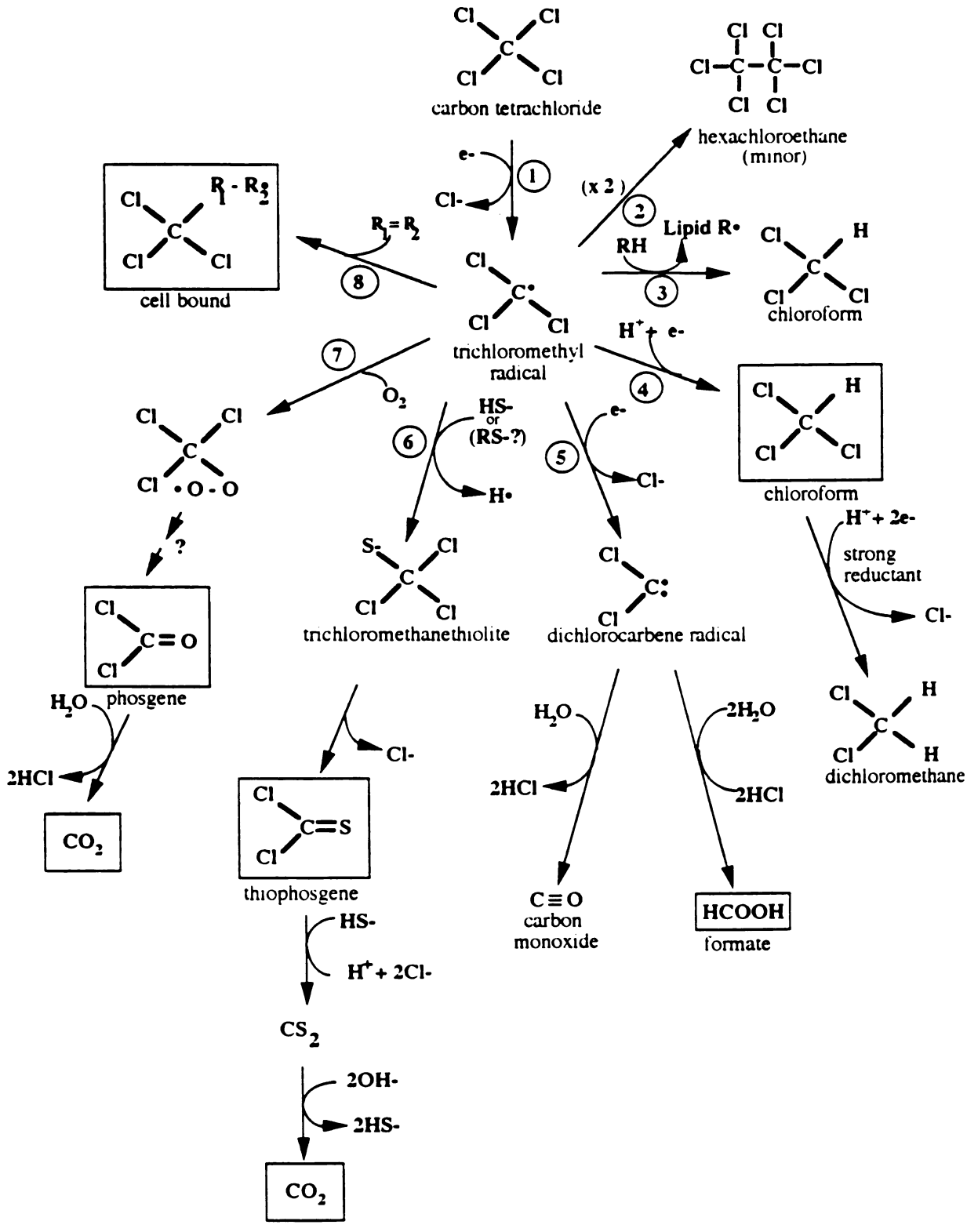

Figure 1.1. Known transformation pathways of CCl_4. Individual pathways are indicated by the circled numbers. Transformation products produced by *P. stutzeri* KC are shown in boxes. Adapted from Criddle and McCarty [26], Kreigman-King and Reinhard[33], Jain and Criddle [27], and Lewis and Crawford [39].

Pathway 1: Trichloromethyl radical formation

Transformation of CCl_4 to the trichloromethyl radical proceeds by the following reaction:

$$CCl_4 + e^- \longrightarrow \cdot CCl_3 + Cl^- \qquad (1.1)$$

The formation of the trichloromethyl radical is generally accepted as the rate limiting step in reductions of alkyl halides [6,54]. The electron can come from one of several reduced species, including certain transition metals, organics, enzymes and co-factors, or it can be omitted by a cathode. Direct evidence of trichloromethyl radical formation comes from time resolved pulse radiolysis conductivity experiments and chloride-ion product analysis [32]. Trichloromethyl radical formation has also been detected using spin trapping techniques [37]. The trichloromethyl radical formed from this step is extremely reactive, giving rise to the broad product distributions observed for CCl_4 transformations proceeding through this step (Figure 1.1).

Pathway 2: Dimerization of the trichloromethyl radical to give hexachloroethane

Evidence for hexachloromethane production from CCl_4 comes from observations that rabbits which breathed CCl_4 exhaled small amounts of hexachloroethane [22]. The production of hexachloroethane also provides indirect evidence of radical formation.

$$2[\cdot CCl_3] \longrightarrow C_2Cl_6 \qquad (1.2)$$

Pathway (3): Interaction with a lipid to form chloroform

The formation of chloroform from CCl_4 was first reported by Butler [9], who observed the production of chloroform in animals given CCl_4. Later studies showed that this biotransformation involved microsomal P-450 dependent enzymes. Several researchers have suggested that the conversion of CCl_4 to chloroform in microsomes requires the intermediate formation of the trichloromethyl radical via a one-electron process. [50,22]. Furthermore, abstraction of a methylene hydrogen by the trichloromethyl radical would account for both the formation of chloroform and the initiation of lipid peroxidation. In order to determine the source of the hydrogen atom in chloroform, Kubic and Anders [36] studied the enzymatic conversion of CCl_4 to chloroform by microsomal P-450 dependent enzymes in the presence of deuterium oxide. They observed no enrichment in deuterium when microsomes from rats were employed, indicating that the source of hydrogen was from lipids and proteins. Luke et al. [40], observed that the trichloromethyl radical could abstract hydrogen if the carbon-hydrogen bond strength was less than 9.2 kcal/mole, which implied that in microsomal systems, the hydrogen would have to come from the unsaturated region of lipid molecules or from localized environments where the resulting carbon radical is stabilized. Therefore, the production of chloroform involves hydrogenation of the trichloromethyl radical by reaction 1.3.

$$\bullet CCl_3 + RH \longrightarrow CHCl_3 + R\bullet \qquad (1.3)$$

Pathway (4): Sequential reduction to chloroform and dichloromethane

Chloroform is one of the most recalcitrant one-carbon alkyl halide, and because of its persistence, (hydrolysis half life of 1850 years at 25 °C; [29]), toxicity, and carcinogenicity, it is one of the least desirable metabolites of CCl_4 transformations.

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Chloroform is produced from CCl_4 by hydrogenolysis-- a reductive dehalogenation in which hydrogen replaces chlorine. The hydrogenolysis of CCl_4 is a two-step process: in the first step, a chlorine is removed by reaction 1.1; in the second step, the trichloromethyl radical is hydrogenated by reaction 1.4.

$$\bullet CCl_3 + H^+ + e^- \longrightarrow CHCl_3 \qquad (1.4)$$

Table 1.1 summarizes the available information on the hydrogenolysis of CCl_4 in microbial systems. Generally, faster and more extensive hydrogenolysis occurs as the environment becomes more reducing--- the highest rate is observed under a methanogenic condition, the next highest under sulfate respiring, and lower rates are observed for fumarate respiring and fermenting conditions.

Table 1.1. Kinetics and conditions of CCl_4 transformation

Culture Type	k' (L/mg protein/day)	Mediated By	Products	Reference
Shewanella putrefaciens MR-1	Fe 3+ 0.177± 0.057 Nitrate 0.105 ± 0.039 TMAO 0.053 ± 0.018 Fumarate 0.029 ± 0.004	anaerobic respiratory components, cytochrome *c*	$CHCl_3$, CO_2, cell bound material, unknowns	45
Desulfobacterium autotrophicum	0.39	acetyl CoA pathway	CO_2, $CHCl_3$, CH_2Cl_2, cell material, unknowns	20,21
Methanobacterium thermoautotrophicum	0.019	acetyl CoA pathway	CO_2, $CHCl_3$, CH_2Cl_2, CH_4, cell material	11, 19
Methanosarcina barkeri	0.873	acetyl CoA pathway F430	$CHCl_3$, CH_2Cl_2, CO_2, CH_4, unknowns	34
Acetobacterium woodii	0.24	acetyl CoA pathway	$CHCl_3$, CH_2Cl_2, CO_2,cell material unknowns	21
Clostridium thermoaceticum	Not Determined[a]	acetyl CoA pathway	$CHCl_3$, CH_2Cl_2, CH_3Cl, unknowns	21
Clostridium sp. strain TCAIIB	0.181	unknown	CHCl3, CH_2Cl_2 unknowns	23
Escherichia coli K12	0.0041 fermenting 0.0025 fumarate respiring	unknown, possibly cytochromes	$CHCl_3$, cell material unknowns	14

As shown in Figure 1.1, dichloromethane is produced by the further reduction of chloroform. Numerous isolates have been obtained which are capable of this reduction (Table 1.1). Gossett [25] reported that chloroform disappeared in methanogenic mixed cultures: 31% of the chloroform was converted to dichloromethane, and a small fraction was further converted to chloromethane; 32-34% was further converted to CO_2. As the degree of chlorination decreases in the series from CCl_4 to chloromethane, removal of

chlorine substituents by reduction becomes energetically and kinetically more difficult. This is true of alkyl halides in general [52]. In the case of microbial hydrogenolysis, experimental observations tend to corroborate theoretical predictions; i.e. chloroform reduction to dichloromethane and dichloromethane reduction to chloromethane are only known to occur in highly reduced environments [53].

The frequent persistence of CCl_4 in microbial systems should also be emphasized. Egli et al. [21] reported that neither *Desulfobacter hydrogenophilus* nor a hydrogen-oxidizing, nitrate-reducing autotrophic bacterium from a groundwater treatment plant could degrade CCl_4. Several denitrifying enrichments from Moffett Field, California, were incapable of CCl_4 transformation as well [28]. Additionally, Bouwer and McCarty [7] reported no CCl_4 transformation for an aerobic mixed culture derived from a sewage inoculum.

In addition to the microbial transformations listed above, several *in vitro* transformation systems have been described. Butler [9] reported that high concentrations (50 mM) of glutathione, cysteine, and ascorbic acid were all capable of reducing CCl_4 to chloroform. Yeast extract with sulfide has been shown to reduce CCl_4 to chloroform [24]. A combination of cytochrome *c* (0.8 mM) and ascorbic acid (5 mM) also brought about conversion of CCl_4 to chloroform. In addition, the reduction of CCl_4 to chloroform is observed in many complex mammalian systems [9, 22, 41], iron porphyrin-sulfide systems [30], and electrolytic systems [13]. Gantzer and Wackett [23] demonstrated the reductive dechlorination of CCl_4 by bacterial transition-metal coenzymes vitamin B_{12} (Co), coenzyme F_{430} (Ni), and hematin (Fe). Rates of reductive dechlorination catalyzed by the co-enzymes decreased markedly with decreasing chlorine content of the alkyl halide.

In summary, reduction of CCl_4 to chloroform occurs when reductants of sufficient reducing power are present and competitive oxidants are not. As the environment becomes more reduced, the rate of chloroform production increases, as does the likelihood of further reduction to dichloromethane. Under highly reducing conditions (as in methanogenic cultures) it may be possible to drive the reductive dehalogenation of CCl_4 past chloroform to dichloromethane, chloromethane, CH_4, and CO_2.

Pathway (5): Second one-electron reduction to form a dichlorocarbene radical

Dichlorocarbene ($:CCl_2$) is produced from chloroform under alkaline conditions by the reactions 1.5a and 1.5b [26]. In theory, it is also possible for dichlorocarbene to be produced by a two-electron reduction of CCl_4, as shown in reaction 1.5c [3]. Dichlorocarbene is known to hydrolyze to give carbon monoxide (CO) and/or formic acid by reactions 1.5d and 1.5e [30; 43].

$$CHCl_3 + OH^- \longrightarrow CCl_3^- + H_2O \qquad (1.5a)$$

$$CCl_3^- \longrightarrow :CCl_2 + Cl^- \qquad (1.5b)$$

$$CCl_4 + 2e^- \longrightarrow :CCl_2 + 2Cl^- \qquad (1.5c)$$

$$:CCl_2 + H_2O \longrightarrow CO + 2HCl \qquad (1.5d)$$

$$:CCl_2 + 2H_2O \longrightarrow HCOOH + 2HCl \qquad (1.5e)$$

The observed formation of CO_2 under anaerobic conditions may be explained by the oxidation of a carbenoid or by the oxidation of CO or formate. The term "carbenoid" is used when the existence of free carbenes is uncertain [43]. Thus, carbenoids may be free or bound in a chloromethyl complex. Carbenoids or chloromethyl complexes that are known or believed to participate in CCl_4 transformations are: chromium carbenoids, iron-porphyrin complexes, cytochrome P-450 complexes, and cobalt corrinoids. An

explanation and evidence for each of these species is described in the following paragraphs.

Castro and Kray [10] reported the Cr(II) sulfate converts CCl_4 to CO (75%) and CH_4 (25%) in a 1:1 dimethylformamide:water solution. They hypothesized that this transformation proceeded via a sequence of reactive halocarbene intermediates.

The formation of carbenoids by iron center species, such as iron-porphyrins and cytochrome P-450 is debated. Brault et al. [8] observed the formation of a dichlorocarbene complex by reacting CCl_4 with reduced deuterohemin. Subsequent oxidation of the complex by molecular oxygen gave CO_2 and small amounts of CO. Mansuy [42] reported a method for preparation of iron-porphyrin carbene complexes, and hypothesized the formation of cytochrome p-450 carbene complexes in the metabolism of polyhalogenated compounds. Klecka and Gonsior [31], in their study of iron-porphyrin reactions with CCl_4 and other alkyl halides, could account for only 31% of the initial CCl_4 added as chloroform produced, and postulated that the missing CCl_4 may have formed a dichlorocarbene complex.

Wolf et al. [55] reported CO formation by rat liver microsomes. They postulated that a carbene or carbenoid species was an intermediate in this reaction. Ahr et al. [2] also reported CO production in microsomal systems. They reported that the reaction was dependent upon cytochrome P-450 and a source of reducing equivalents (NADH or sodium dithionite). DeGroot and Haas [16] reported formation of limited CO in rat liver microsomes, and found that microsomal CO production was inhibited by oxygen. They found evidence to suggest that CCl_4 conversion to CO in microsomes was destructive process in which CCl_4 metabolites inactivated the cytochrome P-450. However, Castro et al. [12] argued that chloroform was the only major product of cytochrome P-450. They

attributed observations of CO production by cytochrome P-450 to the incorrect use of alkaline conditions, resulting in the hydrolysis of CF to CO by reactions 1.5 a b and d. Thus, the direct conversion of CCl_4 to a dichlorocarbene radical in biotic systems remains controversial, and can only theorized to occur based on observed end-products.

Pathway (6): Reaction of trichloromethyl radical with HS-

The first report of CS_2 as a CCl_4 transformation product was in systems with fumarate-respiring and fermenting *Escherichia coli* [14]. In the fumarate respiring and fermenting *E. coli* systems, CS_2 was a minor product (4.3% and 1.6% of the added CCl_4 respectively). Subsequently, Kreigman-King and Reinhard [33] reported the disappearance of CCl_4 and the appearance of products in an abiotic heterogeneous system containing biotite and 1 mM HS-. Carbon disulfide (CS_2) was identified as a major intermediate of this transformation. Studies on the hydrolysis and oxidation of CS_2 show that CS_2 is hydrolyzed to CO_2 by hydroxide ion (OH^-). Adewuyi and Carmichael [1] proposed the following steps for CS_2 hydrolysis:

$$CS_2 + OH^- \longrightarrow CS_2OH^- \text{ (slow)} \qquad (1.6a)$$

$$CS_2OH^- + OH^- \longrightarrow CSO_2H^- + HS^- \qquad (1.6b)$$

$$CSO_2H^- + OH^- \longrightarrow CO_3H^- + HS^- \qquad (1.6c)$$

where the hydrolysis of CS_2 to dithiocarbonate (CS_2OH^-) is the rate limiting step. Assuming CS_2 is stoichiometrically converted to CO_2, ~85% of the CCl_4 is ultimately transformed to CO_2 in these systems [1].

The only pathway previously hypothesized to form CO_2 in anaerobic systems is direct hydrolysis, however, there has been no evidence that CCl_4 can undergo direct hydrolysis

at appreciable rates [29]. Kriegman -King [33] reported that CS_2 appears to be a major intermediate which is transformed to CO_2. They hypothesized that CS_2 may form via nucleophilic substitution of CCl_4 by aqueous or absorbed HS^- or by S_x^{2-}. The S_N2 reactivity of a compound with four identical leaving groups on the same carbon is quite low. Therefore, it is conceivable that CCl_4 accepts one electron to form a trichloromethyl radical, which can react in one of the following ways: (1) it can react with HS^- and release H•; (2) it can first accept another electron to form the trichloromethyl anion which then reacts with S_x^{2-}. or $S_2O_3^{2-}$ to release S_{x-1}^{2-} or SO_3^{2-}, respectively; or; (3) it can first react with S_x^{2-}. or $S_2O_3^{2-}$ producing S_{x-1}^{2-}- or SO_3^{2-} respectively, and a trichloromethyl radical which then accepts an electron. These proposed pathways are illustrated below as reactions 1.6d, e and f. These three proposed pathways all result in the formation of trichloromethanethiolate (CCl_3S^-). S_x^{2-}. and $S_2O_3^{2-}$ are likely to be present in these systems described by Kreigman -King and Reinhard [33] from the reaction of HS^- and ferric-ion in minerals. CCl_3S^-, the proposed intermediate that could form by either the nucleophilic substitution pathway or the electron-transfer-pathway, should decompose to form thiophosgene ($Cl_2C{=}S$), which is transformed to CO_2. As discussed above, CS_2 is ultimately hydrolyzed to CO_2 by OH-. In addition, it is possible that rather than by reacting with OH^- to form CO_2, thiophosgene could react with water to form CO_2 via a carbonyl sulfide (COS) intermediate. However, this intermediate was not observed in the abiotic transformation system described by Kreigman-King and Reinhard [33].

$$\bullet CCl_3 + HS^- \longrightarrow CCl_3S^- + H\bullet \qquad (1.6d)$$

$$\bullet CCl_3 + S_2O_3^{2-} + e^- \longrightarrow CCl_3S^- + SO_3^{2-} \qquad (1.6\,e)$$

$$\bullet CCl_3 + S_x^{2-} + e^- \longrightarrow CCl_3S^- + S_{x-1}^{2-} \qquad (1.6f)$$

Pathway (7) Addition of oxygen to the trichloromethyl radical

Molecular oxygen (O_2) adds to the trichloromethyl radical to give a peroxy radical by the reaction:

$$\bullet CCl_3 + O_2 \longrightarrow CCl3OO\bullet \qquad (1.7a)$$

The mechanism by which peroxy radicals (as shown in the above reaction) are converted to CO_2 is uncertain. In one proposed pathway, peroxy radicals abstract hydrogen from an organic compound in the environment, leading to the production of trichlorohydroperoxide (1.7b). Trichlorohydroperoxide hydrolyzes releasing H_2O_2 and phosgene (1.7c-[4]). In the second pathway, two peroxy radicals combine, releasing molecular oxygen and two alkoxy radicals (1.7d). The alkoxy radicals cleave homolytically to give a chlorine radical and phosgene (1.7e) [44], or they are reduced to give trichloromethanol (1.7f) [5], which decomposes to phosgene (1.7g) Phosgene formed by 1.7 c,e, or g hydrolyzes to CO_2 as shown in reaction 1.7 h.

$$CCl_3OO\bullet + RH \longrightarrow CCl_3OOH + R\bullet \qquad (1.7b)$$

$$CCl_3OOH + H_2O \longrightarrow O{=}CCl_2 + H_2O_2 + HCl \qquad (1.7c)$$

$$2[CCl_3OO\bullet] \longrightarrow 2CCl_3O\bullet + O_2 \qquad (1.7d)$$

$$CCl_3O\bullet \longrightarrow O{=}CCl_2 + Cl\bullet \qquad (1.7e)$$

$$CCl_3O\bullet + H^+ + e^- \longrightarrow CCl_3OH \qquad (1.7f)$$

$$CCl_3OH \longrightarrow O{=}CCl_2 + HCl \qquad (1.7g)$$

$$O{=}CCl_2 + H_2O \longrightarrow CO_2 + 2HCl \qquad (1.7h)$$

In the above reactions, phosgene ($O{=}CCl_2$) is an expected intermediate in the aerobic transformation of CCl_4 to CO_2. Shah et al. [46] and Kubik and Anders [35] reported the

formation of phosgene from CCl_4. Kubik and Anders [35] used ^{18}O labeled O_2 to demonstrate that oxygen incorporated into phosgene was derived from O_2 and not from water.

Phosgene acylates amines, including amino acids such as cysteine as shown in reaction 1.7i. The reactivity of phosgene with cysteine led to the development of means trap phosgene, thereby proving its role as an intermediate in the aerobic transformation of CCl_4 to CO_2 in rat liver homogenate [46].

$$O{=}CCl_2 + R\text{-}NH_2 \longrightarrow O{=}CCINH\text{-}R \qquad (1.7i)$$

Given the transformation of CCl_4 in aerobic mammalian and abiotic systems, it is puzzling that aerobic transformations of CCl_4 by microbial systems are not well documented. One reason aerobic transformations may not occur is that oxygen can act as a competitive inhibitor with CCl_4 for electrons, thus blocking the fist step of the reaction which is the formation of a trichloromethyl radical. Competition for electrons may explain the lack of CCl_4 transformation in aerobic mixed cultures observed by Bouwer and McCarty [7].

Pathway 8: Covalent binding of the trichloromethyl radical to cell material

The binding of trichloromethyl radicals to cell material is well documented, and represents a significant portion of transformed CCl_4 products in many systems. Trudell et al. [49] demonstrated that the trichloromethyl radical reacts with the double-bonds of phospholipids. Trichloromethyl radicals also react with other double bonds--typically attaching to the carbon with the most hydrogen substituents [43]. Covalently-bound CCl_4-metabolites were reported by Ahr et al. [2] for microsomal systems. Most of the

reported metabolites bound to lipids rather than proteins. DiRenzo et al. [17] reported that covalent binding of trichloromethyl radicals to the lipids and proteins of rat hepatocytes was greater under anoxic conditions. Sipes et al. [47] found that the binding of trichloromethyl radicals to rat liver microsomes was three times greater under a nitrogen atmospheres rather than under an atmosphere of air. This increase in cell bound material under anoxic conditions is likely due to the competition between O_2 and cell material for trichloromethyl radicals.

Literature Summary

From the above literature summary, the unifying aspect of CCl_4 transformation is the initial generation of a trichloromethyl radical; subsequent transformations occur by competing pathways in both biotic and abiotic systems. However, a few general conclusions can be made about these pathways. In anoxic systems, faster and more extensive reductive dechlorination generally occurs as the environment becomes more reducing--highest rates are generally obtained under methanogenic conditions, next highest under sulfate-respiring, and slower rates observed under fumarate respiring and fermenting conditions. If the environment is sufficiently reducing, the chloroform produced can be further reduced to dichloromethane or chloromethane. Transformations in the presence of HS- or RS- holds promise for abiotic transformations that do not produce chloroform. However, these transformations are typically slow. Alternately, the competition for electrons by other oxidants such as molecular oxygen may reduce the efficiency of electron transfer to CCl_4, thus blocking the fist step of the reaction which is the formation of a trichloromethyl radical.

***Pseudomonas stutzeri* KC**

In 1988, Criddle et al. obtained a natural aquifer isolate that was capable of rapidly transforming CCl_4 to CO_2 (45-55%), a cell-associated fraction (5%), and a remaining unidentified non-volatile fraction (40-50%) under denitrifying conditions [15]. Immediately, it was apparent that the mechanism of transformation by this organism was not easily understood in the context of previously characterized transformations. This was the first report of a microbial transformation of CCl_4 that did not result in appreciable chloroform production under anoxic conditions. The rates observed for CCl_4 transformation under denitrifying conditions by *P. stutzeri* KC [15] were greater than those previously observed under methanogenic conditions [34], thus disputing the original conclusion that rates of CCl_4 transformation increase as the environment becomes more reduced. The inhibition of CCl_4 transformation under aerobic conditions argued against the formation of CO_2 by a phosgene intermediate. Initially, it was not even possible to conclude that the transformation occurred by the generation of a trichloromethyl radical.

Since the original report by Criddle et al. [15], much work has focused on the elucidation of the mechanism of CCl_4 transformation by *P. stutzeri* KC. In Figure 1.1, the determined products and intermediates of CCl_4 transformation by *P. stutzeri* KC are boxed. Originally, Criddle et al.[15] determined that CO_2, unidentified non-volatiles, and cell-bound material were produced from the anoxic transformation with little or no chloroform production. Lewis and Crawford [38], reported that approximately 5% of the originally added CCl_4 was converted to chloroform by anoxic cultures of strain KC. Dybas et al [18] determined that 20% of the initially added CCl_4 was converted to formate. More recently, Lewis and Crawford [39] identified thiophosgene and phosgene

condensation products, suggesting that these compounds are intermediates in the transformation of CCl_4 by *P. stutzeri* KC.

The transformation products and reaction intermediates identified suggests a very complex picture for CCl_4 transformation by *P. stutzeri* KC. The production of formate requires a two-electron transfer to the CCl_4 molecule, resulting in the formation of a dichlorocarbene radical as illustrated in Figure 1.2. This radical can subsequently be hydrolyzed to formate and CO. In the report by Dybas et al. [18], CO was not tested as a product of transformation. Additionally, the observation of formate production by Dybas et al. does not address the large proportion of CCl_4 that is converted to CO_2 by strain KC. Alternatively, Lewis and Crawford identified condensation products that are typically produced following the reaction of either thiophosgene with DMED or phosgene with cysteine (Figure 1.3). Additionally, a HEPES condensation product was identified as resulting from the interaction of the buffer with thiophosgene. The identification of these products led Lewis and Crawford to conclude that phosgene and thiophosgene are intermediates in the transformation, and that the transformation occurs via a one-electron reduction pathway. However, their conclusions leave many questions unanswered. The production of phosgene requires the addition of O_2 in the system. Therefore, it is very difficult to explain CO_2 production by this pathway under anoxic conditions. Secondly, the conversion of thiophosgene to CO_2 is very slow, as previously discussed in section 6 of this chapter. Additionally, these pathways proposed by Lewis and Crawford do not take into account the production of formate reported by Dybas et al.

It may be possible to bring some unity to this transformation mechanism if phosgene and thiophosgene are formed from the dichlorocarbene radical as shown in Figure 1.4. There currently exist no direct experimental evidence for this pathway, but there also exists no evidence to suggest that this pathway is <u>not</u> theoretically possible. A thermodynamic

analysis indicates that reduction of water by dichlorocarbene to form phosgene and H_2 has an $E^{o'}$ value of +0.66 V. Although this reaction is thermodynamically favorable, further evidence is required to either prove or disprove this pathway, as currently there is not enough data to support either a one- or two-electron reduction pathway for CCl_4 transformation by *P. stutzeri* KC.

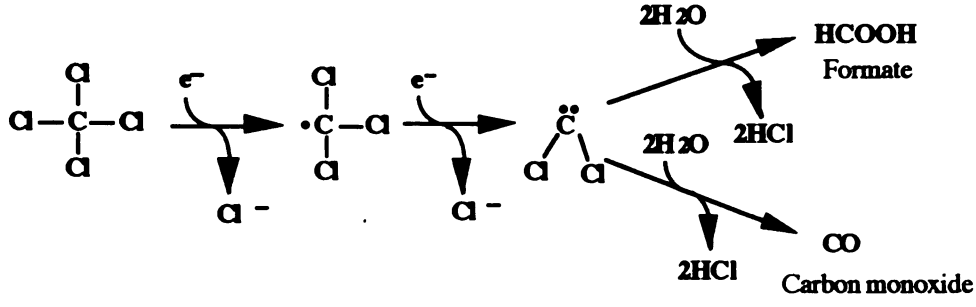

Figure 1.2. Dichlorocarbene intermediate pathway

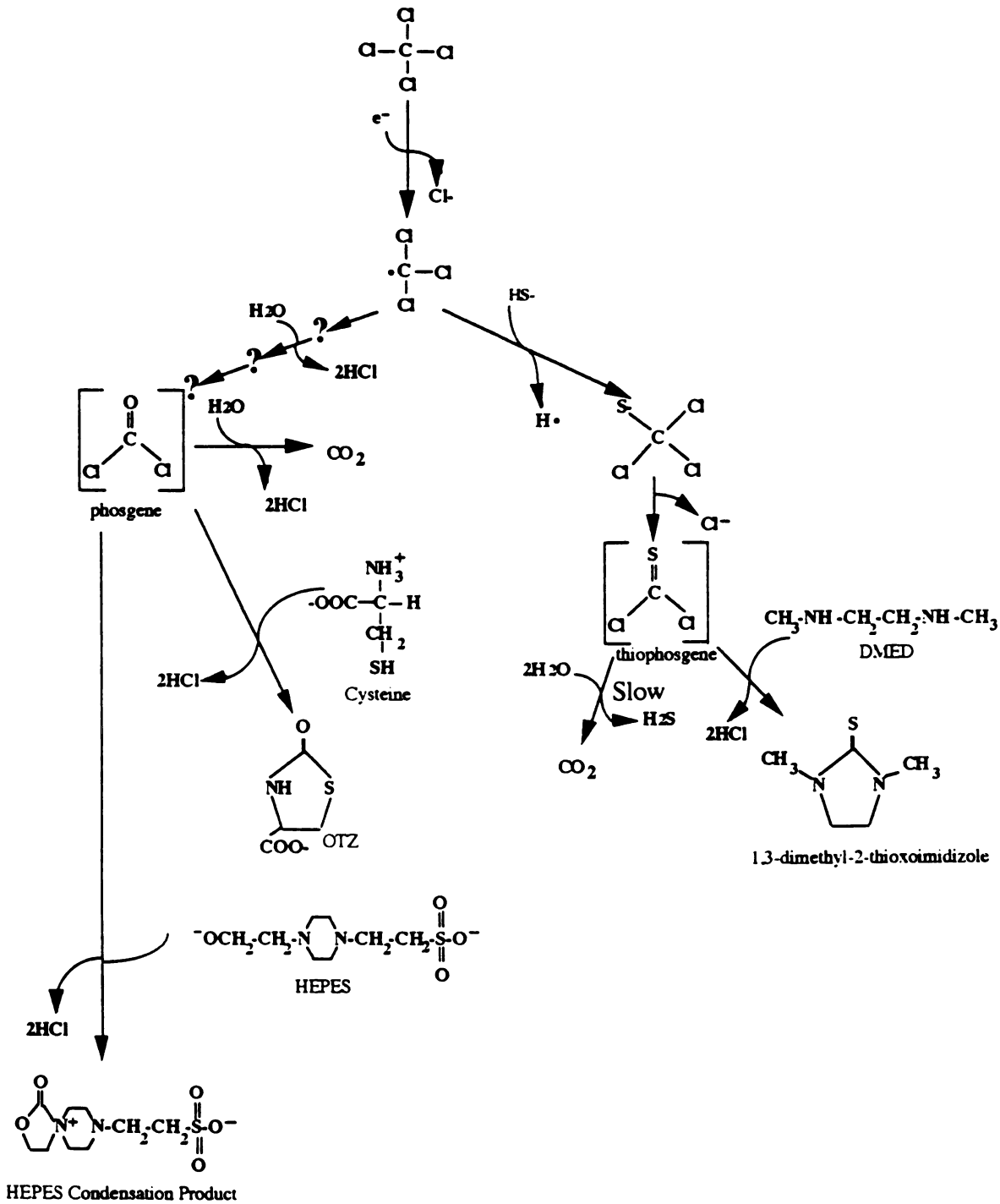

Figure 1.3 Condensation products observed by Lewis and Crawford[38]

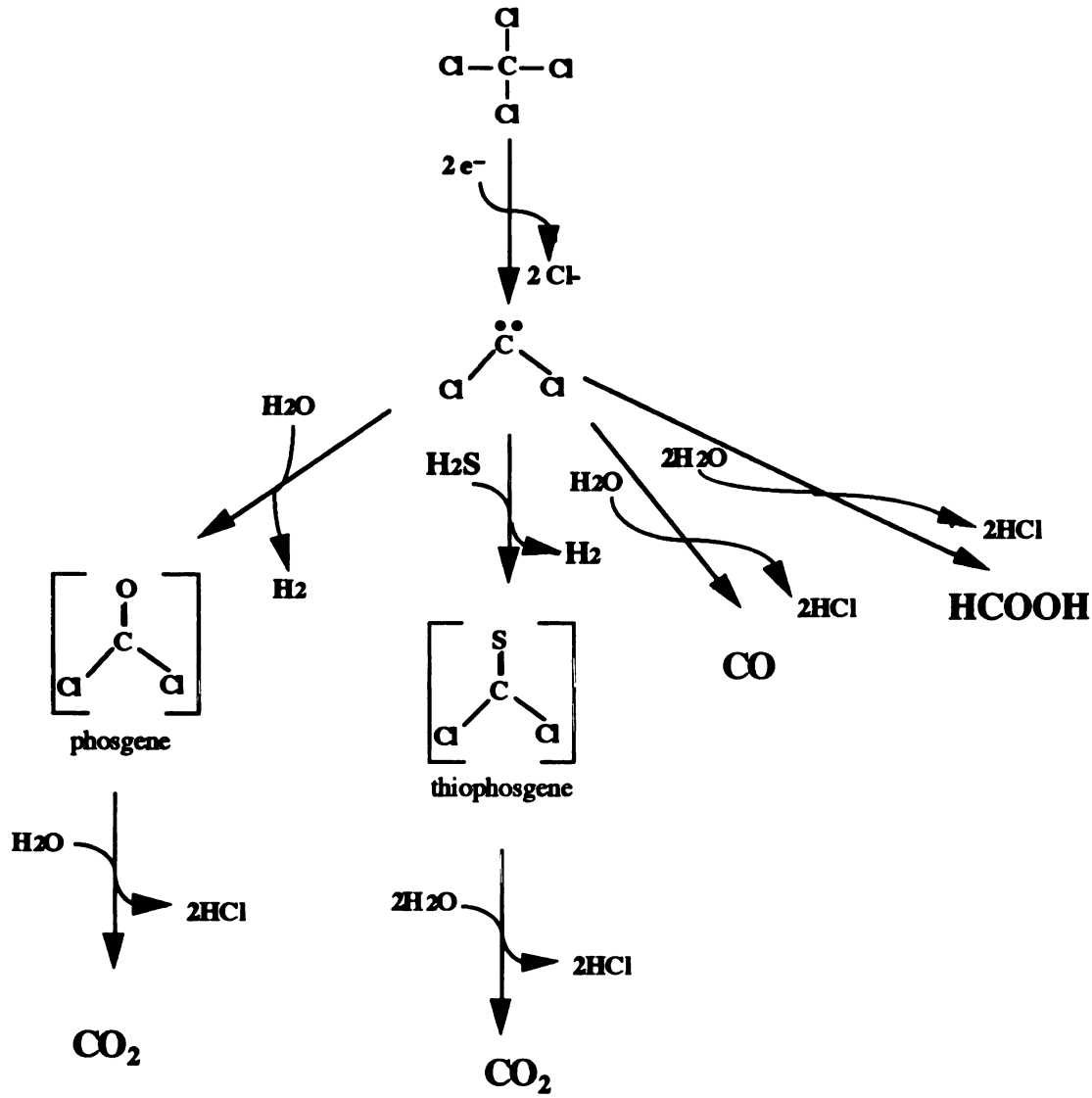

Figure 1.4. Proposed phosgene and thiophosgene intermediate pathway resulting from a dichlorocarbene radical.

Criddle et al. [15] reported that the mechanism for transformation by *P. stutzeri* KC appeared to be linked to the trace metal- or iron-scavenging functions of the cell. This observation provided the underlying hypothesis for the studies presented in this thesis which states that the iron scavenging or siderophore system of *P. stutzeri* is responsible for the cometabolism of CCl_4. It is my rational that identification of the biochemical components and processes responsible for CCl_4 transformation provides the most

appropriate means to elucidate the mechanism(s) and pathway(s) of CCl_4 transformation. by *P. stutzeri* KC.

OUTLINE OF THIS THESIS

The following chapters describe my studies on the CCl_4 transformation system of *P. stutzeri* KC . In Chapter 2, I examine CCl_4 transformation kinetics with respect to CCl_4 concentration, determine the effects of iron and copper on CCl_4 transformation, and determine the fate of ^{14}C-CCl_4 in cultures of *P. stutzeri* KC. These studies were combined with transformations of CCl_4 in groundwater and soil systems conducted by Michael Dybas, and published in *Appl. Env. Microb.* **59:** 2126-2131 1993. In chapter 3, I present the discovery that both extracellular and cellular factors from *P. stutzeri* KC are required for CCl_4 transformation. The extracellular factor is shown to be less than 500 MW, and preliminary purification by acetone extraction and HPLC is also described. These results were combined with product identification studies performed by Michael Dybas and published in *Appl. Environ. Microbiol.* **61:** 758-762, 1995. The combination of the secreted factor(s) produced by strain KC with a diverse range of cell types to result in CCl_4 transformation is described in chapter 4. This discovery led to the development of a bioassay which enabled me to determine: (1) the pH optimum of CCl_4 transformation, (2) the reversible inhibition of CCl_4 transformation by O_2, and (3) the transformation of CCl_4 by indigenous microorganisms in soil slurries amended with a secreted factor preparations. Portions of this chapter were published in Bioremediation Series, 3(3) Bioremediation of Chlorinated Solvents, p. 69-77. In chapter 5, I describe the role of membranes in CCl_4 transformation. The possible engineered application of *P. stutzeri* KC using vegetable oils as a growth substrate is described in chapter 6 and the production of chloroform as an end product of CCl_4 transformation is demonstrated under these growth conditions. Finally, in chapter 7, I summarize my results on CCl_4 transformation and present a model that summarizes my current understanding of CCl_4 transformation by *P. stutzeri* KC and aids in facilitating future studies.

REFERENCES

1. **Adewuyi, Y.G., , G.R. Carmichael.** Kinetics of hydrolysis and oxidation of carbon disulfide by hydrogen peroxide in alkaline medium and apllication to carbonyl sulfide. *Environ. Sci. Technol.* 1987, 21, 170-177.

2. **Ahr, H.J., L.J. King, and W. Nastainczyk, and V. Ullrich.** 1980. The mechanism of chloroform and carbon monoxide formation from tetrachloromethane by microsomal cytochrome P-450. *Biochem. Pharmacol.* **29:** 2855-2861.

3. **Anders, M.W.**,1982. Aliphatic halogented hydrocarbons. Chapter 2 in: *Metabolic Basis of Detoxication: Metabolism of Gunctional Groups.* W.B. Jakoby, J.R. Bend, and J. Caldwell, ed., Academic Press, New York, NY.

4. **Asmus, K.D., D. Bahnemann, K. Krischer, M. Lal, and J. Monig**. 1985. One -electron induced degradation of halogenated methanes and ethanse in oxygenated and anoxic aqueous solutions. *Life Chemistry Reports* **3:** 1-15.

5. **Bahnemann, D. W., C.H. Fischer, M.R. Hoffmann, A.P. Hong, J. Monig, and C. Kormann.** 1987. Mechanistic study of photocatalytic decomposition of organic compounds on semiconductor particles, Preprint extended abstract. Presented at the Meeting of the Division of Environmental Chemistry, American Chemical Society, New Orleans, Louisiana, Aug 30-Sept 4, 1987.

6. **Bakac. A. and J.H. Espenson**. 1985. Kinetics and mechanism of the alkylnickel formation in one-electron reductions of alkyl halides and hydroperoxides by a macrocyclic nickel (I) complex. *J. Am. Chem. Soc.* **108**: 713-719.

7. **Bouwer, E.J. and P.L. McCarty** 1983a. Transformations of 1- and 20 carbon halogenated aliphatic organic compounds under methanogenic conditions, **App. Env. Micro. 45(4)**:1286-1294.

8. **Brault, D., P. Morliere, and M. Rougee**. 1978. Action du tetrachlorure de carbone et du chloroforme sur les hemes, en relation avec le role du cytochrome p450 dans le metablisme et l'hepatotoxicite des composes polyhalogenes. *Biochimie* **60.** 1031-1035.

9. **Butler. T.C.** 1961 Reduction of carbon tetachloride in vivo and reduction of carbon tetrachlroide and chlroform in vitro by tissues and tissue constituents. J. *Pharmaco. Exp. Theory.* **134:** 311

10. **Castro, C.E. and W.C. Kray**. 1966. Carbenoid intermediates from polyhalomethanes and chromium(II). *J.Am. Chem. Soc*. **88(19)**: 4447-4455.

11. **Castro, C.E., M.C. Helvenston, and N.O. Belser.** 1994. Biodehalogenation, reductive dehalogenation of by *Methanobacterium thermoautotrophicum*. Comparison with nickel(I)octoethylisobacteriochlorin anion. An F430 model. *Environ. Tox. and Chem*. **13:** 429-433.

12. **Castro, C.E., R.S. Wade, and N.O. Belser**. 1985. Biodehalogenation; reactions of cytochrome p-450 with polyhalomethanes. *Biochemistry* **24**: 204-210.

13. **Criddle, C.S. and P.L. Mc Carty.** 1991. Electrolytic model system for reductive dehalogenation in aqueous environments. *Environ. Sci. Technol.* **25:** 973-978.

14. **Criddle, C. S., J. T. DeWitt, and P. L. McCarty.** 1990. Reductive dehalogenation of carbon tetrachloride by *Escherichia coli* K-12. Appl. Environ. Microbiol. **56:**3247-3254.

15. **Criddle, C. S., J. T. DeWitt, D. Grbić-Galić, and P. L. McCarty.** 1990. Transformation of carbon tetrachloride by *Pseudomonas* sp. strain KC under denitrification conditions. *Appl. Environ. Microbiol.* **56**:3240-3246.

16. **DeGroot, H. and W. Haas,** 1981. Self-catalyzed, O_2-independent inactivation of NADPH- or dithionite-reduced microsomal cytochrome p-450 by carbon tetrachloride. *Biochem. Pharm.* **30(16)**: 2343-2347.

17. **DiRenzo, A.B., J.J. Gandolfi, I.G. Sipes, and K. Brendel**. 1984. Effect of O_2 tension on the bioactivation and metabolism of aliphatic halides by primary rat-hepatocyte cultures. *Xenobiotica* **14(7)**: 521-525.

18. **Dybas, M.J., G. M. Tatara, and C.S. Criddle.** 1994. Localization and characterization of the carbon tetrachloride transforming activity of *Pseudomonas* sp. strain KC. *Appl. Environ. Microbiol.* **61:** 758-762.

19. **Egli, C., R. Scholtz, A. M. Cook, and T. Leisinger.** 1987. Anaerobic dechlorination of tetrachloromethane and 1,2-dichloromethane to degradable products by pure cultures of *Desulfobacterium* sp. and *Methanobacterium* sp. *FEMS Microbiol. Lett.* **43:** 257-261.

20. **Egli, C., S. Stromeyer, A. M. Cook, and T. Leisinger.** 1990. Transformation of tetra- and trichloromethane to CO_2 by anaerobic bacteria is a non-enzymatic process. *FEMS Microbiol. Lett.* **68:** 207-212.

21. **Egli, C., T. Tschan, R. Scholtz, A. M. Cook, and T. Leisinger**. 1988. Transformation of tetrachloromethane to dichlormethane and carbon dioxide by *Acetobacterium woodii*. *Appl. Environ. Microbiol.* **54:** 2819-2824.

22. **Fowler, S.J. L.** 1969. Carbon tetrachloride transformation in the rabbit. *Br. J. Pham.* **37:** 733 - 737

23. **Gantzer, C.J. and L.P. Wackett.** 1991. Reductive dechlorination catalyzed by bacterial transition-metal coenzymes. *Environ. Sci. Technol.* **25**: 715-722.

24. **Galli, R. and P.L. McCarty.** 1989. Biotransformation of 1,1,1-trichloroethane, trichloromethane, and tetrachloromethane by a *Clostridium* sp. *Appl. Environ. Microbiol.* **55:** 837 - 844.

25. **Gossett, J.M.** 1985. Anaerobic degradation of C_1 and C_2 chlorinated hydrocarbons, final report ESL-TR85-88, Air Force Engineering and Services Center, Tyndall Air Force base, 1985.

26. **Hine, J.** 1950. Carbon dichloride as an intermediate in the basic hydrolysis of chloroform. A mechanism, for substitution reactions at the saturated carbon atom. *J. Am. Chem. Soc.* **72**: 2438-2445.

27. **Jain, M.K. and C.S. Criddle.** Metabolism and cometabolism of C-1 and C-2 hydrocarbons. *In* Ved Pal Singh (ed.), Biotransformations: Microbial Degradation of Health Risk Compounds vol. 32. Elsevier Scienc B. V., Amsterdam, The Netherlands.

28. **Jansson, M.** 1987. Anaerobic dissolution of iron-phosphorus complexes in sediment due to the activity of nitrate-reducing bacteria. *Microb. Ecol.* **14:** 81-89.

29. **Jeffers, P.M., L.M. Ward, L.M. Woytowitch, and N.L. Wolfe**. 1989. Homogeneous hydrolysis rate constants for selcted chlorinated methanes, ethanes, ethenes, and propanes. *Env. Sci. Tech.* **23 (8)**: 965-969.

30. **Kirmse, W.** 1971. Carbene Chemistry. Volume 1. Academic Press, New York.

31. **Klecka, G.M. and S.J. Gonsior** 1984. Reductive dechlorination of chlorinated methanes and ethanes by reduced iron(II) porphyrins. *Chemosphere* **13 (3)**: 391-402.

32. **Koster, R. and K.D. Asmus**, 1971. Die reduktion von tetrachlorkohlenstoff durch hydratisierte elektronen, H-atome und reduzierende radikale, *Z. Naturforsch* **26b**, 1104 - 1108.)

33. **Kriegman-King, M.R. and Martin Reinhard**. 1992. Transformation of carbon tetrachloride in the presence of sulfide, biotite, and vermiculite. *Environ. Sci. Technol.* **26:** 2198-2206.

34. **Krone, U.E. and R.K. Thauer.** 1992. Dehalogenation of trichlorofluoromethane (CFC-11) by *Methanosarcina barkeri*. *FEMS Microbiol. Lett.* **90:** 210-204.

35. **Kubik, V.L. and M.W. Anders** 1980. Metabolism of carbon tetrachloride to phosgene. *Life Sciences* **26:** 2151-2155.

36. **Kubik, V.L. and M.W. Anders**. 1981. Mechanism of microsomal reduction of carbon tetrachloride and halothane. *Chem. Biol. Interactions*. **34:** 201-207.

37. **Lai, E.K., P.B. McCay, T. Noguchi, and K.L. Fong,** 1979. In vivo spin-trapping of trichloromethyl radicals formed from CCl4. *Biochem. Pharm*. 28. 2231 - 2235.

38. **Lewis, T. A., and R. L. Crawford**. 1993. Physiological factors affecting carbon tetrachloride dehalogenation by the denitrifying bacterium *Pseudomonas* sp. strain KC. *Appl. Environ. Microbiol.* **59**:1635-1641.

39. **Lewis, T.A. and R.L. Crawford.** 1995. Transformation of carbon tetrchloride via sulfur and oxygen substitution by *Pseudomonas* sp. strain KC. *J. Bact.* 177: 2204-2208.

40. **Luke, B.T., G.H. Loew, and A.D. McClean**. 1987. Theorhetical investigations of the anaerobic reduction of halogenated alkanes by cytochrome p450. 1. Structures, inversion barriers, and heats of formation of halomethyl radical. J. *Am. Chem. Soc.* **109:** 1307-1317.

41. **Macdonald, T.L** 1983. Chemical mechanisms of halocarbon metabolism. *CRC Critical Reviews in Toxiclolgy* **11:** 85-120

42. **Mansuy, D.** 1980. New iron-porphyrin complexes with metal-carbon bond - biological implications. *Pure & Appl. Chem*. **52:** 681-690.

43. **March, J.M.** 1985. Advanced Orgainic Chemistry. John-Wiley & Sons, New York, NY

44. **Monig, J. , D. Bahnemann, and K.D. Asmus,**1983. One electron reduction of CCl4 in oxygenated aqueous solutions: a CCl3O2-free radical mediated formation of Cl- and CO2. *Chem. Biol. Interactions.* **45:** 15-27.

45. **Petrovski, E.A., T.M. Vogel, and P. Adriens.** 1994. Effects of electron acceptors and donors on transformation of tetrachloromethane by *Shewanella putrefaciens* MR-1. *FEMS Microbiol. Lett.* **121:** 357-364.

46. **Shah, H., S.P. Hartman, and S. Weinhous.** 1979. Formation of carbonyl chloride in carbon tetrachloride metabolism by rat liver in vitro. *Cancer Research* **39:** 3942-3947.

47. **Sipes, I.G., G. Krishna, and J.R. Gillette.** 1977. Bioactivaton of carbon tetrachloride, chloroform and bromotrichloromethane: role of cytochrome p-450. *Life Sciences* **20:** 1541-1548.

48. **Sittig, M.** (ed.) 1985. Handbook of Toxic and Hazardous Chemicals and Carcinogens, Second Edition, pp. 194-196, Noyes Publications, New York.

49. **Trudell, J. R., B. Bosterling, and A.J. Trevor.** 1982. Reductive metabolism of carbon tetrachloride by human cytochromes p-450 reconstituted in phospholipid vvescicles: mass spectral identification of trichloromethyl radical bound to dioleoyl phosphatidylcholine. *Proc. Natl. Acad. Sci.* **79:** 2678-2682.

50. **Uehleke, H., K.H. Hellmer, S. Tabarelli.** 1973. Binding of ^{14}C-carbon tetrachloride to microsomal proteins in vitro and formation of CHCl3 by reduced liver microsomes, Xenobiotica, 3: 1

51. **Verschueren K.** (ed) 1983. Handbook of Toxic and Hazardous Chemicals and Carcinogens, Second Edition, pp. 166-168, Van Nostrand Reinhold Company, New York.

52. **Vogel, T.M. and P.L. McCarty**. 1987. Abiotic and biotic transformations of 1,1,1-trichloroethane under methanogenic conditions. *Env. Sci. Tech.* **21:** 1208-1213.

53. **Vogel, T.M., C.S. Criddle, and P.L. McCarty**. 1987. Transformations of halogenated aliphatic compounds, *Env. Sci. Tech.* **21:** 722-736.

54. **Wade, R.S. and C.E. Castro.** 1973. Oxidation of iron (II) porpyrins by alkyl halides. *J. Am. Chem. Soc.* **95 (1):** 226-230.

55. **Wolf, C.R., D. Mansuy, W. Nastainczyk, G. Deutschmann, and V. Ullrich**. 1977. The reduction of polyhalogenated methanes by liver microsomal cytochrome p-450. *Molecular Pharm.* **13:** 698-705.

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CHAPTER 2

EFFECTS OF MEDIUM AND TRACE METALS ON KINETICS OF CARBON TETRACHLORIDE TRANSFORMATION BY *PSEUDOMONAS STUTZERI* KC

These studies were combined with transformations of CCl_4 in groundwater and soil systems performed by Dr. Michael J. Dybas and published in
Applied and Environmental Microbiology, 59: 2126-2131, 1993.

ABSTRACT

Under denitrifying conditions, *Pseudomonas stutzeri* KC transforms CCl_4 to CO_2 via an undetermined mechanism. *P. stutzeri* KC converted ^{14}C-labeled CCl_4 to $^{14}CO_2$ (37%), a non-purgeable filterable fraction (20%), and a non-purgeable non-filterable fraction (34%), with no evidence of interconversion of products. Transformation rates were first-order with respect to CCl_4 concentration over the range examined (0-100 μg/L), and proportional to protein concentration, giving pseudo-second-order kinetics overall. Addition of ferric ion (1-20 μM) to an actively transforming culture inhibited CCl_4 transformation, and the degree of inhibition increased with increasing iron concentration. By removing iron from the trace metals solution or by removing iron-containing precipitate from the growth medium, higher second-order rate coefficients were obtained. Copper also plays a role in CCl_4 transformation. Copper (1μM) was toxic to strain KC cells at neutral pH. By adjusting media pH to 8.2, soluble iron and copper levels decreased as a precipitate formed, and CCl_4 transformation rates increased. However, cultures grown at pH 8.0 without 1 μM copper added to the growth medium exhibited slower growth rates and greatly reduced rates of CCl_4 transformation, indicating that copper is required for CCl_4 transformation.

INTRODUCTION

In recent years, considerable interest has surrounded prospects for degrading hazardous contaminants by stimulating selected bacterial populations (biostimulation) or by addition of novel organisms to contaminated sites (bioaugmentation). Stimulation of an indigenous population is likely to yield an enrichment that is well adapted to its environment, whereas foreign organisms introduced into such an environment may be unable to compete. However, introduced organisms do offer certain advantages provided they can compete with the indigenous microorganisms. Unlike indigenous organisms, introduced organisms can be extensively studied and understood in the laboratory, improving prospects for control of their activity in the field. Among other reasons, control of activity is needed to avoid the production of unwanted by-products. Chloroform, for example, is a common end product of CCl_4 transformation in both laboratory and field environments [2, 4, 5, 7]. chloroform is more persistent than CCl_4 in many environments, and it is also a suspected carcinogen. Consequently, metabolic pathways that do not produce chloroform are of interest. *Pseudomonas stutzeri* KC is an aquifer-derived organism that transforms CCl_4 to CO_2 and unidentified non-volatile products without chloroform production under denitrifying conditions [3]. Related *Pseudomonas* species grown and assayed under the same conditions do not transform CCl_4. In the following sections I report on: (1)the quantification of end products of CCl_4 transformation, (2) the kinetics of CCl_4 transformation by *P. stutzeri* KC, and (3) the role of iron and copper in CCl_4 transformation kinetics.

MATERIALS AND METHODS

Chemicals. CCl_4 (99+% purity) was obtained from Aldrich Chemical Co., Milwaukee, Wisconsin. ^{14}C-labeled CCl_4 (250 μCi @ 99% purity) with a specific activity of 4.3 mCi/mmol was obtained from NEN Research Products. A ^{14}C-labeled CCl_4 stock solution in isooctane was prepared as per Criddle et al. (1990). All chemicals for media preparation were ACS reagent grade (Aldrich or Sigma Chem. Co.), and all water used was 18 megaohm resistance or greater.

Media preparation and growth conditions. Medium D [3] contained (per liter of de-ionized water) 2.0 g of KH_2PO_4, 3.5 g K_2HPO_4, 1.0 g of $(NH_4)_2SO_4$, 0.5 g of $MgSO_4 \cdot 7H_2O$, 1 milliliter of trace nutrient stock TN2, 1 milliliter of 0.15M $Ca(NO_3)_2$, 3.0 g of sodium acetate, and 2.0 g of sodium nitrate. Stock solution TN2 contained (per liter of deionized water) 1.36 g of $FeSO_4 \cdot 7H_2O$, 0.24 g of $Na_2MoO_4 \cdot 2H_2O$, 0.25 g of $CuSO_4 \cdot 5H_2O$, 0.58 g of $ZnSO_4 \cdot 7H_2O$, 0.29 g of $Co(NO_3)_2 \cdot 6H_2O$, 0.11 g of $NiSO_4 \cdot 6H_2O$, 35 mg of Na_2SeO_3, 62 mg of H_3BO_3, 0.12 g of NH_4VO_3, 1.01 g of $MnSO_4 \cdot H_2O$, and 1 ml of H_2SO_4 (concentrated). Some experiments used different trace metal preparations to study their effects on CCl_4 transformation. TN2-Cu and TN2-Fe stock solutions lacked $CuSO_4$ and $FeSO_4$ respectively, but were otherwise identical to TN2. After addition of all essential media components, medium D was adjusted to a desired initial pH of 8.0 or 8.2 with 3 N KOH. This final adjustment in pH resulted in the formation of a white precipitate. The resulting medium was autoclaved at 121 °C for 30 minutes and transferred to an anaerobic glove box (Coy Laboratories, Ann Arbor, Mich.).

Precipitate-free medium D was prepared as follows: medium D (prepared as previously described) was transferred to an anaerobic glove box for quiescent settling of precipitate, and decanted after 24 hours. The precipitate and oxygen-free decanted medium was re-

autoclaved to assure sterility and cooled before use. Precipitate-free medium D contained 24 mM acetate, 25 mM PO_4^{3-}, 19 mM NO_3^-, determined using a Dionex model 2000i-SP ion-chromatography system, and 3.8 nM iron determined using a Perkin Elmer model 1100 graphite furnace atomic absorption spectroscopy system.

Cultures were grown under a N_2 atmosphere in one of three different containers: (1) 28-mL serum tubes (Bellco Glass No. 2048-00150), (2) a modified one-liter Wheaton Bottle as described by Balch and Wolfe [1], and (3) 250-mL (8 oz.) bottles sealed with screw-cap Mininert valves (Alltech catalog number 95326). Both the serum tubes and the modified Wheaton bottles were sealed with teflon-faced butyl rubber septa (West Catalog number 1014-4852) and aluminum crimp seals. All cultures were shaken at 100-150 rpm at 20-23 °C. Strain KC did not transform CCl_4 at temperatures above 25°C, and it did not grow at temperatures above 30°C (data not shown). Culture manipulations were typically performed in an anaerobic glove box under an atmosphere of 98% N_2 and 2% H_2. Oxygen level was monitored continuously with a Coy gas detector model no. 10. Hungate technique was used for anaerobic manipulations outside the glove box.

Analytical methods. All bottles used to evaluate CCl_4 transformation were sealed with pressure tested screw-cap Mininert valves or Teflon-lined butyl rubber stoppers. CCl_4 was assayed by removing 0.1 mL of headspace gas with a 0.25 or 0.5 mL Precision gas-tight syringe (Alltech catalog no. 050032), and injecting the sample into the GC. For μg/L concentrations, the GC was a Perkin Elmer model 8500 equipped with a 100/120 mesh column (10% Alltech CS-10 on Chromsorb W-AW, Alltech Catalog # 12009 PC) and an electron capture detector with nitrogen carrier (40 mL/min) and nitrogen make-up (27 mL/min). For mg/L concentrations, the GC was a Hewlett Packard 5890 gas chromatograph operated isothermally at 150°C and equipped with a DG 624 column

(J&W Scientific Catalog # 125-1334) and a flame ionization detector (hydrogen flowrate = 100 mL/min, air flowrate = 250 mL/min). The carrier gas was nitrogen (16 mL/min).

External standard calibration curves were prepared by addition of a primary standard (7.8 ng CCl_4 per μL methanol or 0.82 μg CCl_4 per μL methanol) to secondary standard water solutions having the same gas/water ratio, ionic strength, incubation temperature, and speed of shaking as the assay samples. A four point calibration curve was prepared over a concentration range bracketing that of the assay samples. Protein was stored by freezing at -20°C, and assayed using the modified Lowry method, with bovine serum albumin as the standard [6].

Mass transfer rates were estimated for 28 mL serum tubes containing 10 mL of medium D and sealed with teflon-lined rubber septa. CCl_4 was added to the aqueous phase, and the tubes were shaken at 100 rpm on a shaker table. Headspace CCl_4 was assayed every 60 seconds for 10 minutes by GC. The mass transfer rate k_La (h^{-1}) was determined by plotting $\ln\left(1 - \frac{C_g}{C_g^e}\right)$ versus shaking time, where C_g = headspace CCl_4 concentration and C_g^e = headspace CCl_4 concentration at equilibrium. The slope of the resulting plot is $-k_La\left(\frac{V_g}{V_{aq}} + \frac{1}{H_c}\right)$, where H_c = dimensionless Henry's constant (1.0 for CCl_4 at 20°C), V_{aq} = water volume and V_g = headspace volume.

Radioisotope experiments. To assess the time course of ^{14}C-CCl_4 transformation, a method of quenching the reaction was needed. In separate experiments, hydrogen peroxide was evaluated and found suitable for this purpose. Hydrogen peroxide halted CCl_4 transformation, did not itself transform CCl_4, and did not alter the distribution of CCl_4 transformation products.

A time series experiment with radiolabeled CCl_4 was conducted using a 2.18 L glass reaction vessel. The vessel was modified so that access to its contents was only possible through two threaded glass openings. These openings were sealed with screw-cap teflon Mininert valves (individually pressure tested). The vessel was filled with 2.17 L of medium D, then inoculated with a 0.15% solution of stationary phase *P. stutzeri* KC, and incubated at room temperature for 120 hours. After the culture had grown to an OD_{660} of 0.75, the bottle was spiked with the ^{14}C-CCl_4 solution in isooctane, and transferred to a rotary shaker for vigorous agitation (150 RPM). After 15 minutes and at regular intervals thereafter, 5 mL of culture was withdrawn using a 5 mL Hamilton gas-tight syringe (Alltech catalog no. 81530). One mL was injected into a scintillation vial containing 10 mg (23 μL) of hydrogen peroxide, 9 mL scintillation cocktail was then added, and the vial was sealed to prevent loss of volatile ^{14}C-labeled CCl_4. A second milliliter of sample was injected into a scintillation vial containing 10 mg (23 μL) of H_2O_2 and 35 μL of 3 N KOH. A third milliliter was injected into a scintillation vial containing 10 mg (23μL) of H_2O_2 and 30 μL of 6 N HCl. A final milliliter of sample was filtered through a 0.2 μm nylon filter into a scintillation vial containing 10 mg (23 μL) H_2O_2 and 30 μL of 6 N HCl. The acid and base samples were purged for 25 minutes with N_2 gas at a flow rate of 10 mL/min. After purging, 9 mL of scintillation cocktail (Beckman Cat. No. 158726) was added. All samples were counted for 5 minutes on a Packard tri-carb liquid scintillation counter (Model 1500). Because ^{14}C was added to the culture in isooctane, a portion of the ^{14}C added to the reaction flask was present in the isooctane phase during the reaction. The partition coefficient for CCl_4 in a water/isooctane system (i.e., dpm/mL of the isooctane divided by dpm/mL of the equilibrated aqueous phase) was measured separately, and a value of 1032±98 was obtained.

Disappearance of CCl_4 and appearance of products were quantified with a simple model in which transformation activity is assumed to decay with time:

$$\frac{dS}{dt} = kSe^{-k_d t} \qquad (1)$$

where S= CCl_4 concentration (mg/L), k=first order rate coefficient (h^{-1}), kd = first-order decay coefficient (h^{-1}). Equation 1 is integrated to obtain S as a function of time:

$$S = S^{\circ}\exp\left[\frac{k}{k_d}(\exp(-k_d t) - 1)\right] \qquad (2)$$

where S°= initial CCl_4 concentration(mg/L).

Formation of products (P) were modeled by using equation (2) assuming that a fraction of S was converted to the products P:

$$P = \delta(S^{\circ} - S) \qquad (3)$$

where P= product concentration. Data were evaluated using a least squares fit of the data to equations 1-3.

Determination of reaction rate order and transformation capacity. The dependence of CCl_4 transformation rate on CCl_4 concentration was assessed with stationary phase cultures grown 72 hours from a 1% inoculum in both medium D and precipitate-free medium D. Cultures were dispensed into 28-mL serum tubes, and CCl_4 was added (5-100 μg/L). The tubes were then transferred to a shaker table, and headspace CCl_4 was periodically monitored by GC. Reaction rates were calculated using measurements taken after 20 minutes had elapsed to allow sufficient time for equilibration of headspace CCl_4 with the water phase CCl_4. Under these conditions, the mass transfer rate (~25 h^{-1}) was much greater than the reaction rate (~1 h^{-1}). The observed rates were corrected for equilibrium partitioning into the gas phase to obtain the true reaction rates (see Modeling). To evaluate the dependence of CCl_4 transformation rate on total culture

protein, a stationary phase culture was diluted 1:5, 1:3, and 1:2 with media, then monitored for CCl_4 removal by sampling of the gas phase.

To determine the transformation capacity of KC cultures grown under different conditions, 100 mL of stationary phase culture (grown for 72 hours in 1 liter modified Wheaton bottles in either medium D or precipitate-free medium D) was dispensed into 170-mL serum vials sealed with teflon-lined septa. The vials were then spiked with CCl_4 to give initial concentrations of 1 mg/L or 5 mg/L, shaken at 100 rpm at 20°C, and monitored for CCl_4 removal by sampling of the gas phase.

Effects of trace metals. To assess the effects of trace copper, medium D was prepared with either stock solution TN2 or TN2-Cu, transferred to 8-oz (250-mL) bottles, sealed, autoclaved, cooled, and inoculated with a 1% (v/v) suspension of a stationary phase culture of *P. stutzeri* KC. Cultures were grown to stationary phase, spiked with CCl_4, and assayed for CCl_4 transformation.

To assess the effects of trace iron, medium D and precipitate-free medium D were prepared using trace metal stock solutions TN2 and TN2-Fe. Cultures were grown 48 or 72 hours, spiked with CCl_4, and assayed for CCl_4 transformation. To investigate iron inhibition, 10 mL of early stationary phase culture (grown for 72 hours in precipitate-free medium D) was transferred to 28 mL serum tubes in an anaerobic glove box, spiked with 0-20 μM ferric iron (as ferric ammonium sulfate), and equilibrated for 10 minutes. The serum tubes were sealed with teflon-lined rubber stoppers, spiked with CCl_4, shaken throughout the experiment, and monitored by sampling of the gas phase.

Modeling. Separate experiments (see Results) established that transformation of CCl_4 was first-order with respect to CCl_4 concentration over the concentration range examined

and first-order with respect to total protein concentration. Assuming a second-order kinetic expression, a mass balance can be written for a closed batch system in which a volatile aqueous phase substrate is in equilibrium with its gas phase:

$$\frac{-dM_{CT}}{dt} = k' C_{aq} X V_{aq} = \frac{k'}{V_{aq}+H_c V_g} M_{CT} X V_{aq} \qquad (1)$$

where: M_{CCl4} = total mass of CCl_4 in the system (mg) = $C_{aq}(V_{aq} + H_c V_g)$, t = time (d), k' = second-order rate coefficient (L/mg protein-d), C_{aq} = aqueous phase concentration of CCl_4 (mg/L), X = concentration of protein (mg/L). Separation of variables and integration of equation 1 yields:

$$\ln\left(\frac{M_{CT}}{M^o_{CT}}\right) = -\left(\frac{k'X\ V_{aq}}{V_{aq}+H_c V_g}\right) t \qquad (2)$$

For a reaction that is first-order with respect to CCl_4 concentration, a plot of the logarithm of the mass of CCl_4 in the bottle vs. time should give a straight line with slope of $k'XV_{aq}/(V_{aq}+H_cV_g)$.

RESULTS

Kinetics of CCl_4 transformation. The dependence of CCl_4 transformation rates on CCl_4 concentration was evaluated by plotting the logarithm of mass of CCl_4 versus time. As shown in Figure 2.1, this plot was linear, and the slopes were essentially constant over the concentration range examined, indicating that the reaction could be represented as first order with respect to CCl_4 concentration.

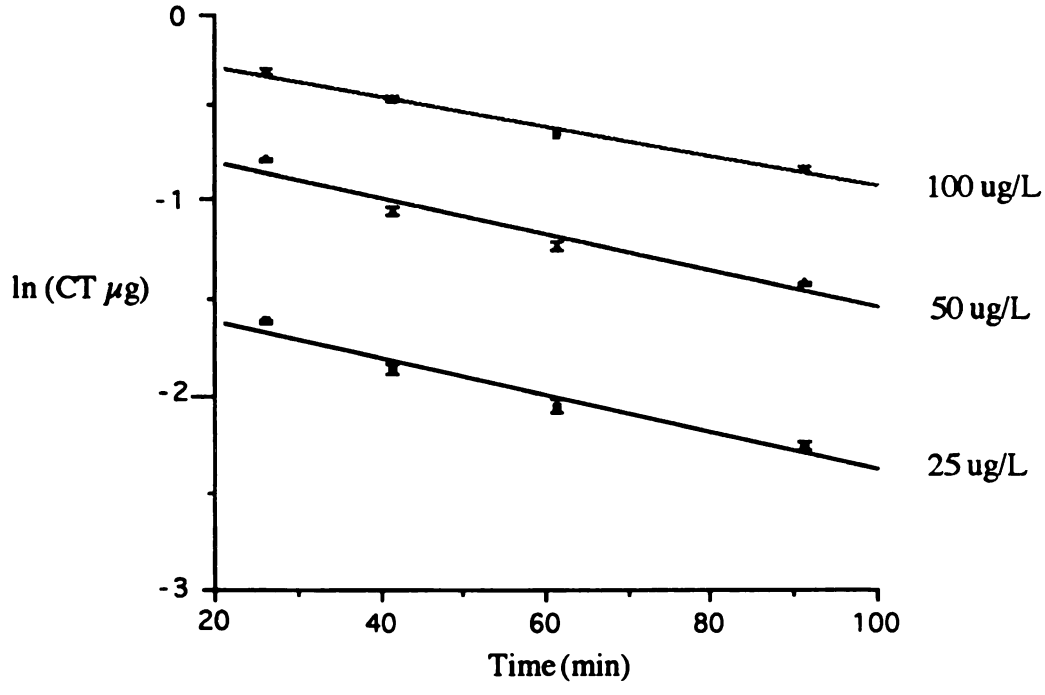

Figure 2.1. Kinetics of CCl_4 loss by strain KC. Slopes for CCl_4 transformation were calculated from natural logarithm of the concentrations 25, 50, and 100μg/L of CCl_4 versus time. Slopes were determined to be 0.0096, 0.0094, and 0.0076 respectively. Error bars representing the standard deviations of four independent samples are generally less than the dimensions of symbols used.

To assess the dependence of the CCl_4 transformation rate on protein concentration, first-order rate coefficients of the culture were plotted against total protein concentration. As shown in Figure 2.2, rates were linearly related to protein concentration range evaluated (6.25-22 μg/mL). Thus, a pseudo-second-order rate expression (first-order in CCl_4 concentration and pseudo-first order in protein concentration) was considered appropriate. Pseudo-second-order rate coefficients were calculated from the slopes of the logarithm of mass versus time plot.

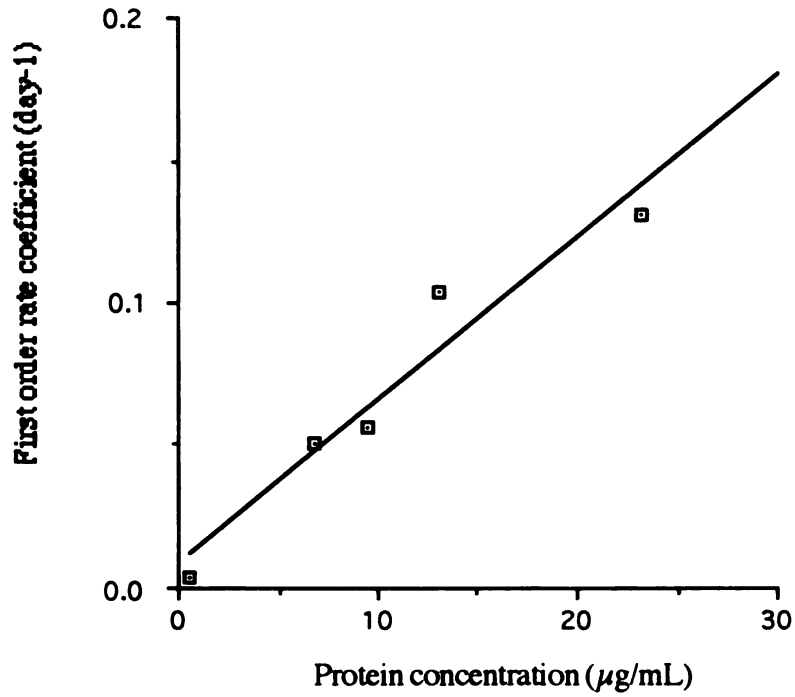

Figure 2.2. Dependence of first-order rate coefficient on total protein concentration.

Fate of ^{14}C-labeled CCl_4. Radiolabeled CCl_4 was rapidly converted to $^{14}CO_2$ (37%), a non-purgeable non-filterable fraction (34%), and a non-purgeable filterable fraction (20%), with no apparent accumulation or interconversion of products (Figure 2.3). ^{14}C-labeled CCl_4 was added as an isooctane stock so partitioning had to be considered. Assuming equilibrium, approximately 17% of CCl_4 was present in isooctane initially, but this fraction dropped to 2% within four hours. Partitioning of CCl_4 into the gas phase was also considered (assuming a dimensionless Henry's constant of 1.0). As liquid volume was removed by sampling, the gas phase volume increased from 0.5% of the reactor volume initially to 6.4% by the end of the experiment (the gas phase was under positive pressure so no vacuum formed during sampling). Assuming equilibrium, the CCl_4 present in the gas phase increased from about 1% of the total CCl_4 present initially to about 8% of the CCl_4 present at the end of the experiment.

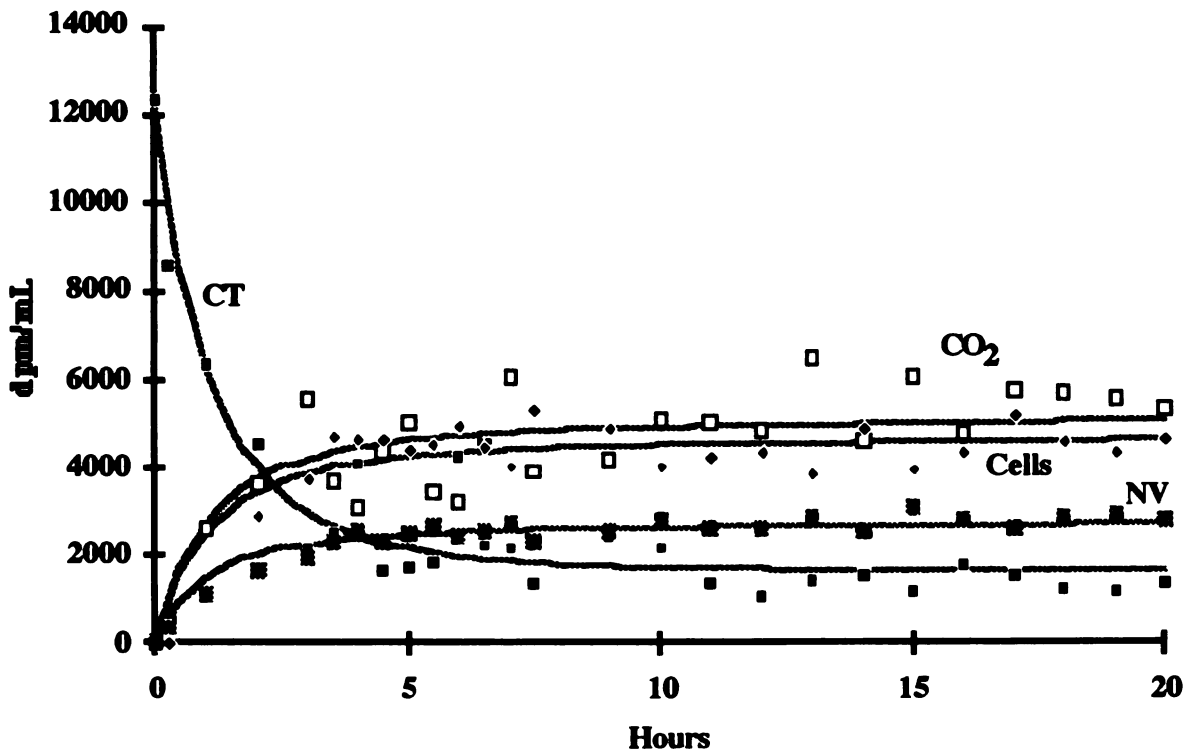

Figure 2.3. Fate of transformed radiolabeled CCl_4 by *P. stutzeri* KC. Lines represent the best fit to the data points as described in materials and methods. k= 0.82 h^{-1}, $\delta_{nv} = 0.200$, $\delta_{cell} = 0.341$, $\delta_{CO_2} = 0.371$, $k_d = 0.41\ h^{-1}$ of equations 2 and 3

Effects of trace metals. Removal of the precipitate that formed during preparation of medium D at pH 8.0 had a profound effect on the iron level of the medium. As determined by atomic adsorption spectroscopy, precipitate-free medium D contained 3.8 nM iron. Removal of iron from the growth medium affected growth rate, protein levels, CCl_4 transformation rate, and CCl_4 transformation capacity. As shown in Figure 2.4, protein concentration at the end of the growth phase was greater for cells grown in medium D (protein concentration = 350 μg/mL) than for cells grown in medium D prepared with TN2-Fe (protein concentration = 51 μg/mL) precipitate-free medium D (protein concentration = 46 μg/mL) and precipitate-free medium D prepared with TN2-Fe (protein concentration = 22.5 μg/mL). These observations indicate that diminished

growth was due to the removal of iron. Cells grown in precipitate-free medium D had higher pseudo-second-order rate coefficients (Table 2.1), but lower 24-hour transformation capacities (Figures 2.5 and 2.6) when compared to medium D. Pseudo-second-order rate coefficients were lowest in the "high" iron media, and highest in the "low" iron media (prepared with TN2-Fe or without precipitate).

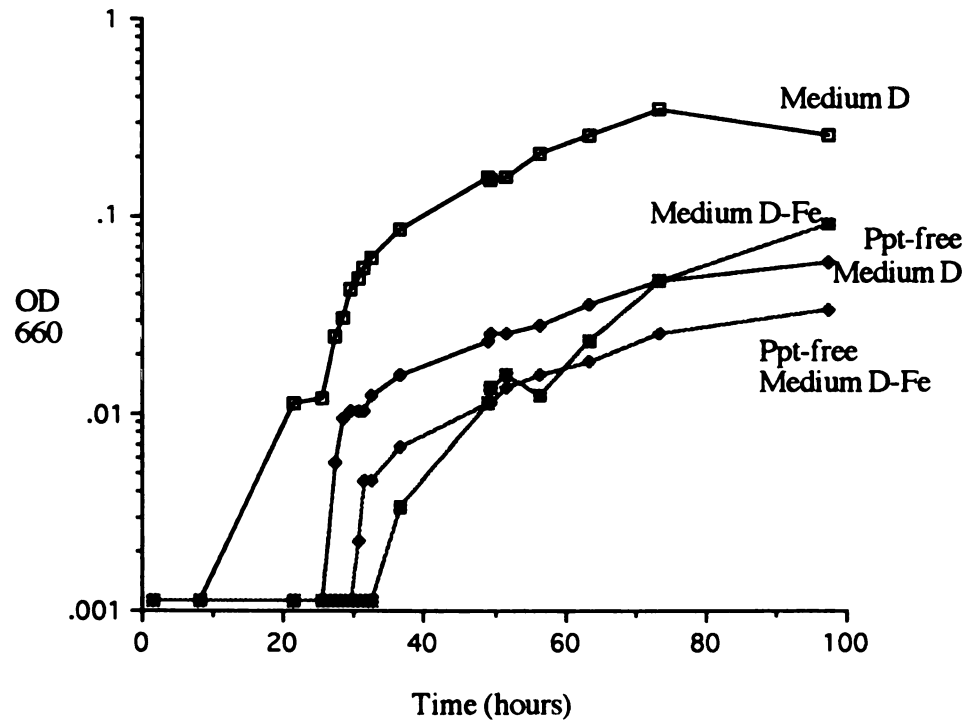

Figure 2.4. Growth of *P. stutzeri* KC in medium D and precipitate-free medium D in the presence or absence of added iron.

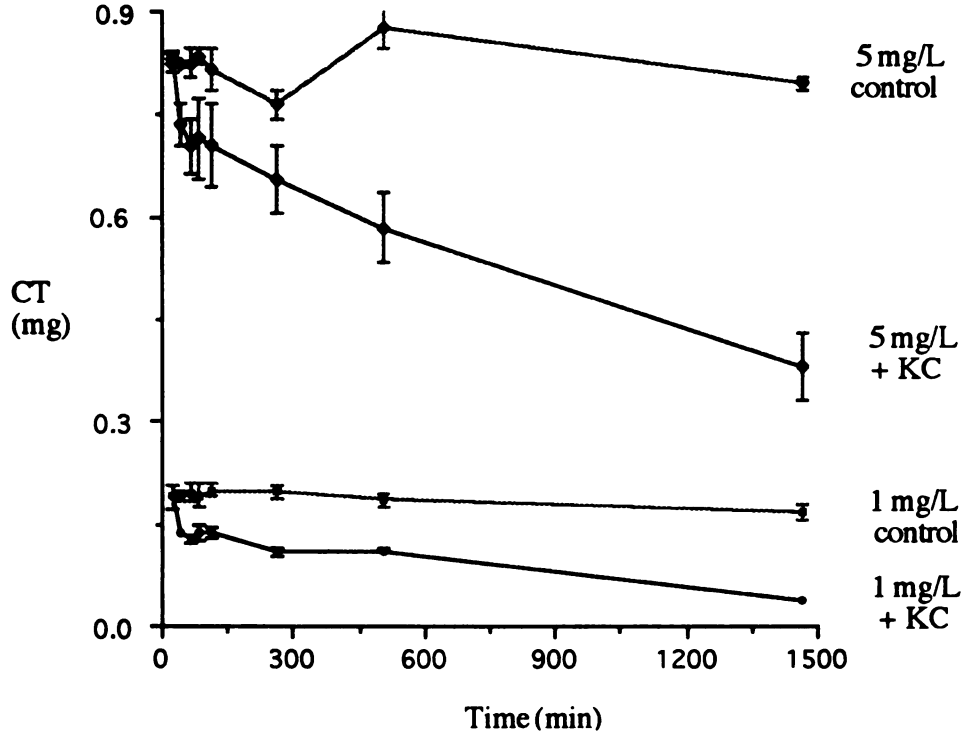

Figure 2.5. CCl_4 transformation capacity for cultures of *P. stutzeri* KC grown in medium D. Concentrations of CCl_4 are given in mg/L are shown on the graph. Error bars represent standard deviations on triplicate samples.

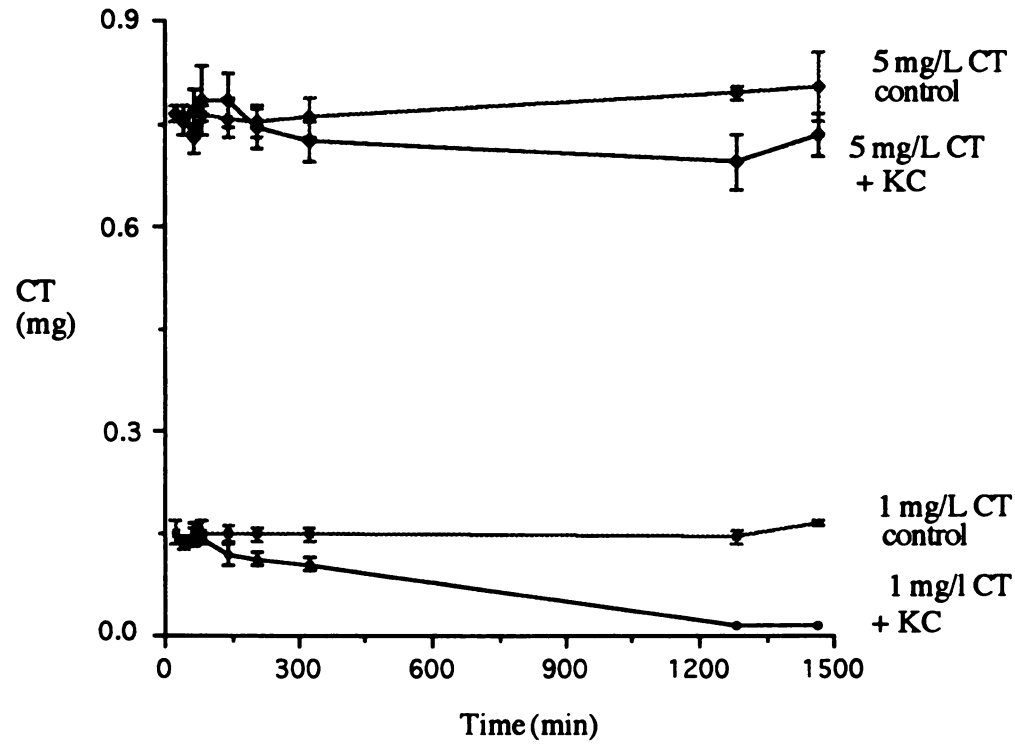

Figure 2.6. CCl_4 transformation capacity for cultures of *P. stutzeri* KC grown in precipitate-free medium D. Concentration of CCl_4 are given in mg/L are shown on the graph. Error bars represent standard deviations on triplicate samples.

As shown in Table 2.1, pseudo-second-order rate coefficients for CCl_4 transformation generally decreased as cultures aged from 48 to 72 hours, indicating decay of transformation activity as cells entered the stationary phase. The exception was cultures grown in medium D with TN2-Fe. These cultures continued to grow between 48 and 72 hours, and showed no decrease in the second order rate coefficient over this period. Growth rates for these cultures were higher and less variable than those of cultures grown in precipitate-free media (Figure 2.4). These observations suggest that, for this medium, cell growth and production of CCl_4 transformation activity may be controlled by the solubilization of iron in the precipitate.

Table 2.1. Second-order rate coefficients (± one standard deviation) for CCl_4 transformation by *P. stutzeri* KC: effects of iron limitation and culture age.

Medium modification	Culture age (hour)	k' (L/mg protein-d)
+ precipitate	48	0.893±0.03
	72	0.362±0.08
+ precipitate - trace Fe	48	3.93±1.48
	72	4.03±0.79
- precipitate	48	6.18±0.48
	72	2.28±0.45
- precipitate - trace Fe	48	9.1±1.2
	72	4.41±0.56

The effect of ferric iron addition to precipitate-free medium D is shown in Figure 2.7. Addition of ferric ammonium sulfate (1, 5, 10, and 20 μM) to an actively transforming stationary phase culture inhibited the rate of CCl_4 transformation CCl_4, and the degree of inhibition increased as the concentration of iron increased.

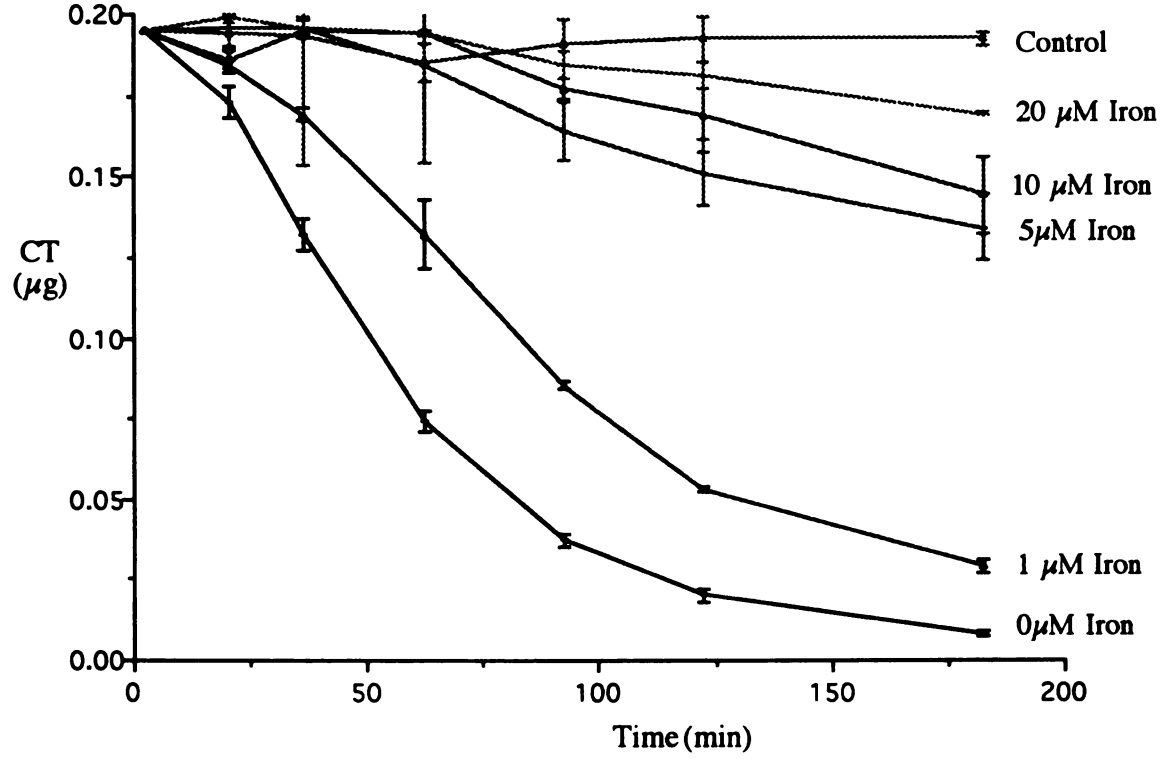

Figure 2.7. Inhibition of CCl_4 transformation by ferric iron. Error bars represent standard deviations on triplicate samples.

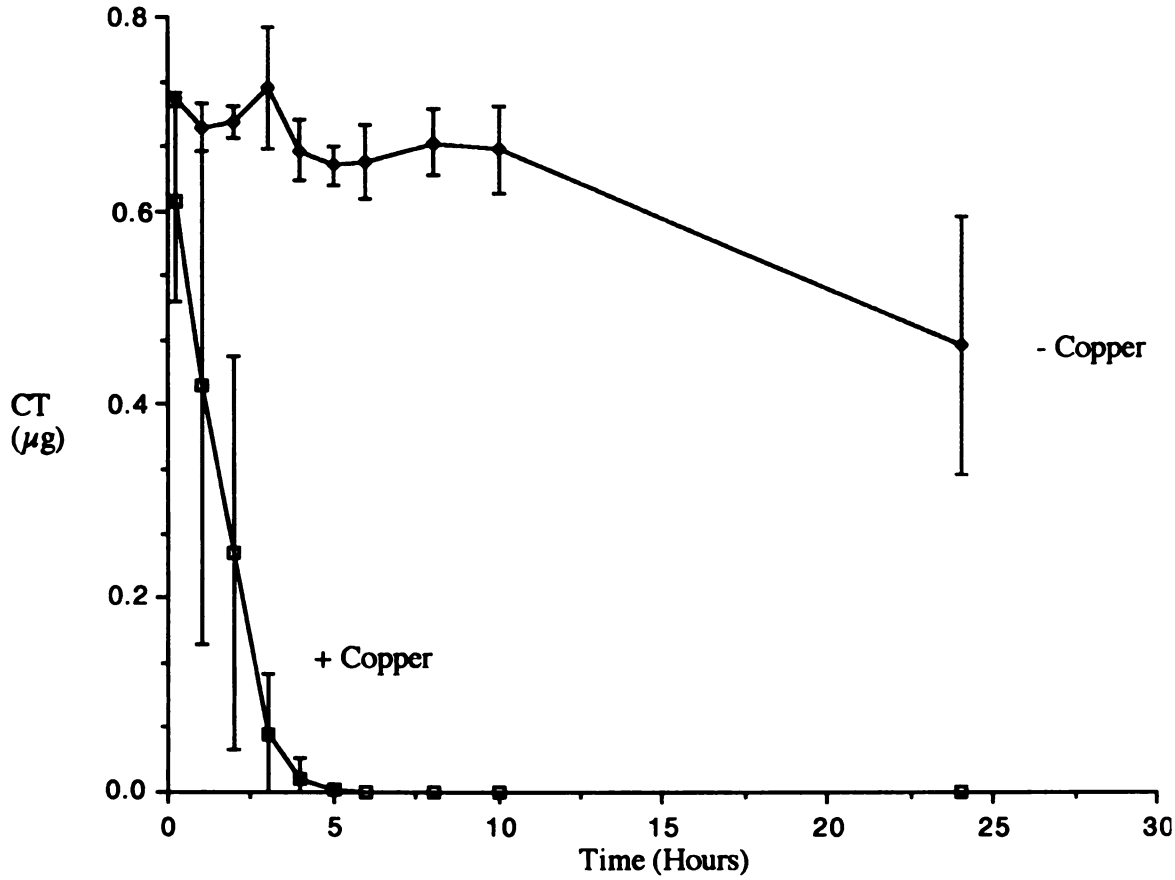

Figure 2.8. Trace levels of copper affect CCl_4 transformation activity. Error bars represent standard deviations on triplicate samples.

Criddle et al. [3] found that 1 μM copper prevented growth of *P. stutzeri* KC at neutral pH. The present work confirmed this finding. In medium D adjusted to pH 8.0, however, the maximum specific growth rate of strain KC decreased in the absence of copper, dropping from 0.047 hr^{-1} for TN2+Cu to 0.016 hr^{-1} for TN2-Cu. Final protein concentration was not greatly affected by copper (185 μg/mL for TN2+Cu compared with 173 μg/ml for TN2-Cu). As shown in Figure 2.8, rapid transformation of CCl_4 was only obtained with TN2+Cu, and little or no transformation of CCl_4 was obtained with TN2-Cu. Thus, omission of only 1 μM copper was sufficient to prevent CCl_4 transformation.

DISCUSSION

My results indicate that transformation of CCl_4 by *P. stutzeri* KC proceeds by a complex mechanism. The transformation appears to be linked to the iron-scavenging functions of the cell, as previously proposed [3]. Observations supporting this hypothesis include the following: (1) KC grown in precipitate-free medium D does not transform CCl_4 if the growth medium is supplemented with trace iron before inoculation with strain KC [3], (2) addition of iron to grown cultures of strain KC inhibits CCl_4 transformation - possibly by competing for a binding site (Figure 2.7), and (3) the second-order rate coefficients for CCl_4 transformation increase for cells grown in iron-limited media (Table 1). Transformation of CCl_4 apparently requires copper and probably involves a reducing agent, as evidenced by the quenching action of oxidants, such as hydrogen peroxide and oxygen (data not shown).

The present work disproves one proposed pathway of CCl_4 transformation. Criddle et al. (1990) hypothesized that CO_2 production from CCl_4 might be explained by the oxidation of the non-purgable non-filterable fraction: a two electron reduction of CCl_4 could produce a dichlorocarbene radical which could spontaneously undergo hydrolysis to form formate; carbon dioxide could subsequently be produced by oxidation of the formate. In the time series experiment with ^{14}C (Figure 2.3), there was no evidence that the end products were interconverted. This observation contradicts the original hypothesis and suggests that transformation proceeds through a rapidly decomposing intermediate which is subsequently transformed via parallel pathways to CO_2, the non-purgeable non-filterable fraction, and a non-purgeable filterable fraction. A less likely explanation is that more than one agent of transformation is operative.

Given the complex mechanism postulated for CCl_4 transformation, it is likely that a complete kinetic description of the transformation will prove equally complex. The agent of transformation must be identified and quantified. More information is needed to understand changes in CCl_4 transformation activity with cell growth stage and trace metal speciation. As shown in Table 2.1, growing cells transform CCl_4 faster than do stationary phase cells. There is also evidence that the CCl_4 transformation is affected by other trace metals, notably cobalt and vanadium [3], and that these trace metals act synergistically with iron to inhibit CCl_4 transformation [Criddle, PhD. Thesis, 1989]. In spite of these complexities, however, the present results do establish a simple first-order dependence on CCl_4 concentration over the CCl_4 concentration range investigated. A pseudo-first-order relationship with total protein concentration was also observed. However, use of total protein in a pseudo-second-order kinetic expression must be viewed as a temporary expediency. Total protein merely functions as a quantifiable surrogate for the actual agent of transformation until such time as the agent itself can be quantified. In the following chapter, I describe studies which further elucidate the components of the CCl_4 transformation system of *P. stutzeri* KC.

REFERENCES

1. **Balch, W. E. and R. S. Wolfe.** 1979. Transport of coenzyme M (2-mercaptoethanesulfonic acid) in *Methanobacterium ruminantium* . *J. Bact.* **137**. 264-273.

2. **Criddle, C. S., J. T. DeWitt, and P. L. McCarty.** 1990. Reductive dehalogenation of carbon tetrachloride by *Escherichia coli* K-12. *Appl. Environ. Microbiol.* **56**:3247-3254.

3. **Criddle, C. S., J. T. DeWitt, D. Grbić-Galić, and P. L. McCarty.** 1990. Transformation of carbon tetrachloride by *Pseudomonas* sp. strain KC under denitrification conditions. *Appl. Environ. Microbiol.* **56**:3240-3246.

4. **Egli, C., R. Scholtz, A. M. Cook, and T. Leisinger.** 1987. Anaerobic dechlorination of tetrachloromethane and 1,2-dichloromethane to degradable products by pure cultures of *Desulfobacterium* sp. *Methanobacterium* sp. F*EMS Microbiology Letters* **43**:257-261.

5. **Egli, C., T. Tschan, R. Scholtz, A. M. Cook, and T. Leisinger.** 1988. Transformation of tetrachloromethane to dichloromethane and carbon dioxide by *Acetobacterium woodii. Appl. Environ. Microbiol.* **54**:2819-2823.

6. **Markwell, M. A., S. M. Haas, N. E. Tolbert, and L. L. Bieber.** 1981. Protein Determination in membrane lipoprotein samples: manual and automated procedures. *Methods Enzymology.* **72**:296-301.

7. **Semprini, L., G. D. Hopkins, P. L. McCarty, and P. V. Roberts.** 1992. In-situ transformation of carbon tetrachloride and other halogenated compounds resulting from biostimulation under anoxic conditions. *Environ. Sci. Technol.* 26:2454-2461.

8. **Sittig, M.** (ed.) 1985. Handbook of Toxic and Hazardous Chemicals and Carcinogens, Second Edition, Noyes Publications, New York.

9. **Stumm and Morgan.** 1981. p. 230-322. *In* Aquatic Chemistry, Second Edition, Wiley and Sons, New York.

CHAPTER 3

LOCALIZATION OF THE CARBON TETRACHLORIDE TRANSFORMATION ACTIVITY OF *PSEUDOMONAS STUTZERI* KC

These studies were combined with end-product identification analyses and protonophore studies performed by Michael J. Dybas and published in
Applied and Environmental Microbiology, 61: 758-762, 1995.

I gratefully acknowledge the co-development of the purification process for the secreted factor by Dr. Michael J. Dybas

ABSTRACT

Previous research has established that rapid transformation of CCl_4 by *Pseudomonas stutzeri* KC requires the organism to be grown under denitrifying and iron-limited conditions. The present study investigated the possible role of iron scavenging agents in the transformation, and presents the finding that both extracellular and intracellular factors are involved in the transformation. By themselves, washed cells of *P. stutzeri* KC did not transform CCl_4 to a significant degree. Occasionally, CCl_4 transformation was observed by cell-free culture supernatant, but this activity was not reliable. Rapid and reliable CCl_4 transformation was only obtained when washed whole cells were reconstituted with culture supernatant, indicating that both extracellular and intracellular factors are normally required for CCl_4 transformation. Fractionation of culture supernatant by ultrafiltration established that the extracellular factor(s) is small, with an apparent molecular weight of less than 500 daltons. Addition of micromolar levels of iron inhibited CCl_4 transformation in whole cultures, but the level of iron needed to inhibit CCl_4 transformation was over one hundred fold higher for washed cells reconstituted with 10,000 dalton supernatant filtrate. Thus, the inhibitory effects of iron are exacerbated by a supernatant factor(s) with a molecular weight greater than 10,000 daltons. The extracellular fraction was further purified following the discovery that it is stable after lyophilization to powder and is extractable with acetone. A fraction containing CCl_4 transformation activity eluted at 27-28 minutes from a semi-preparative reverse phase HPLC column at a flowrate of 7 mL/min with a methanol/water gradient.

INTRODUCTION

Pseudomonas stutzeri KC is a natural aquifer isolate that rapidly transforms CCl_4 to CO_2 and non-volatile end product(s) without the production of chloroform under denitrifying conditions. The ability of *P. stutzeri* KC to transform CCl_4 is dependent on the presence of iron-limiting conditions in the growth medium [1,5,12]. Additionally, ferric iron was shown to inhibit CCl_4 transformation by actively transforming cultures of *P. stutzeri* KC [12]. Based on these observations, it has previously been hypothesized that an iron scavenging system plays a key role in the transformation of CCl_4 by *P. stutzeri* KC [1,12]. In this chapter, I further evaluate the possibility of an iron scavenging system fortuitously transforming CCl_4 by focusing on the role of intracellular and extracellular components involved in transformation activity. I describe studies which demonstrate that an extracellular factor(s) is involved in the transformation and I describe progress made toward partial purification of the extracellular factor(s). Additionally, I characterize the extracellular factor(s) by size and iron sensitivity.

MATERIALS AND METHODS

Organisms. *P. stutzeri* KC (DSM deposit no. 7136, ATTC deposit no. 55595), derived originally from aquifer solids from Seal Beach, CA, [1] is routinely maintained in our laboratories on nutrient agar plates. *Pseudomonas fluorescens* (ATTC deposit no. 13525) was obtained from the culture collection of the Department of Microbiology at Michigan State University.

Chemicals. CCl_4 (99% purity) was obtained from Aldrich Chemical Co., Milwaukee, Wis. All chemicals used were ACS reagent grade (Aldrich or Sigma Chemical Co.). All water used in reagent preparation was 18 Mohm resistance or greater.

Glassware. All glassware used in the purification procedure was acid washed in 6M HCl, rinsed with DI water, rinsed with HPLC grade acetone, and dried at 100°C prior to use in any manipulation.

Media. Medium D was prepared and dispensed in 28 ml serum tubes or modified 1-liter Wheaton bottles as previously described in Chapter 2. Cultures of *P. fluorescens* were grown in Medium D under aerobic conditions. Cultures of strain KC were also grown in medium D at 20°C with 150 rpm shaking under aerobic or denitrifying (N_2 headspace) conditions. For purification of supernatant components, strain KC was grown in simulated groundwater medium SGW (recipe provided by R. Skeen, Battelle Pacific Northwest Laboratory). Medium SGW contained per liter of deionized water: 0.455 g of $Na_2SiO_3 \cdot 9\,H_2O$, 0.16 g Na_2CO_3, 0.006 g of Na_2SO_4, 0.02 g of KOH, 0.118 g of $MgCl_2 \cdot 6H_2O$, 0.0081 g of $CaCl_2 \cdot 2H_2O$, 13.61 g of KH_2PO_4, 1.6 g of NaOH, 1.6 g of $NaNO_3$, 1.6 g of acetate and 1mL of trace element solution. The trace element solution contained per liter of deionized water: 0.021 g of $LiCl_2$, 0.08 g of $CuSO_4 \cdot 5H_2O$, 0.106 g of $ZnSO_4$

· $7H_2O$, 0.6 g of H_3BO_3, 0.123 g of $Al_2(SO_4)_3$ · 18 H_2O, 0.11 g of $NiCl_2$ · 6 H_2O, 0.109 g of $CoSO_4$ · $7H_2O$, 0.06 g of $TiCl_4$, 0.03 g of KBr, 0.03 g of KI, 0.629 g of $MnCl_2$ · $4H_2O$, 0.036 g of $SnCl_2$ · $2H_2O$, 0.3 g of $FeSO_4$ · $7H_2O$. The pH of SGW medium was adjusted to 8.2 with 3 N KOH. The resulting medium was autoclaved at 121°C for 20 minutes then transferred to a Coy anaerobic glove box for degassing.

Analytical methods. CCl_4 was assayed by removing samples of headspace gas above liquid samples and injected the sample onto a gas chromatograph as described previously in Chapter 1. Protein was assayed by the modified Lowry method, with bovine serum albumin as the standard [7].

Bioassay for the secreted factor(s) using *P. fluorescens*. Tatara et al. [13] discovered that rapid CCl_4 degradation occurs when the secreted factor(s) generated by strain KC is combined with diverse cell types, such as cells of *P. fluorescens*. This finding enabled the development of a bioassay for the secreted factor(s). In the bioassay, *P. fluorescens* cells were harvested by centrifugation (12,100 X g, 10 min, 4 °C) and resuspended to one tenth the original culture volume in medium D, to a cell density of approximately 2 x 10^9 cfu/ml. Five hundred microliters of the resulting 10 X concentrated cell suspension was added to 4.5 ml samples generated during the fractionation procedure. The samples were rendered anoxic by passage through the anaerobic interlock on the Coy anaerobic glove box which pulled a vacuum of 20 in. of Hg and replaced the headspace with N_2 nine times. Samples were sealed under a N_2 headspace in 28 ml Balch tubes and spiked with CCl_4. Levels of CCl_4 were followed by headspace gas chromatography as previously described.

Fractionation of CCl_4 transformation activity. To identify the factors involved in CCl_4 transformation activity of strain KC, actively transforming cultures were

fractionated by centrifugation and ultrafiltration. Cultures grown for 24 hours in SGW medium under denitrifying conditions were first screened for CCl_4 transformation activity. Typically, about 300 ml of actively transforming culture was dispensed into degassed Oak Ridge style centrifuge tubes in the anaerobic glove box. This type of centrifuge tube has been shown to exclude oxygen and maintain the strict anaerobic conditions required for methanogenesis during 10 minute centrifuge runs [3]. Cells were harvested by centrifugation (10 minutes at 12,100 X g at 4°C), and the supernatant filtered through a 0.2 μm filter. Occasionally, the cell-free supernatant was capable of CCl_4 transformation. In these instances, it was further fractionated by filtration through Amicon 10,000 and 500 molecular weight cut -off filters in the anaerobic glove box (95% N_2 ± 5% H_2 atmosphere). Filtrate and retentate of each filter were assayed for CCl_4 transformation activity. The CCl_4 transformation assay was performed on 4.5 ml samples of the fractions with and without reconstitution with *P. stutzeri* KC cells or *P. fluorescens* cells. Samples were dispensed under N_2 and spiked with CCl_4 to 10 μg/liter. CCl_4 levels were followed by gas chromatography as described in Chapter 2.

Effects of iron. To assess the effects of iron on CCl_4 transformation, an actively transforming culture was fractionated as described above, individual fractions were spiked with 0-100 μM iron (as ferric ammonium sulfate) and 10 μg/L CCl_4, and each fraction was assayed for CCl_4 transformation. The 10,000 and 500 MW filtrate was tested for activity by recombining (reconstitution) with cells of strain KC as described above.

Siderophore assays. To determine the classes of siderophores produced by strain KC, cultures were fractionated by cell removal and ultra filtration as described above. General iron binding activity of the culture supernatant, 10,000 MW filtrate, 500 MW retentate, and 500 MW filtrate was assayed by the method of Schwyn and Nielands [10].

This universal method to detect siderophores was developed by using their high affinity for iron (III). The ternary complex Chrome Azurol S/iron (III)/hexa-decyltrimethylammonium bromide, with an extinction coefficient of approximately 100,000 M^{-1} cm^{-1} at 630 nm, serves as an indicator. Because of the high sensitivity of this assay, it is useful for the detection of siderophores in supernatants of culture fluids. The C'sasky assay [2] was utilized for the detection of hydroxamate class siderophores, and the Rioux assay [9] was used for the detection of catechol class siderophores using 2,3-dihydroxy benzoic acid as a standard.

Partial acetone purification of transformation activity. Filtrate passed through an Amicon 500 MW filter was lyophilized to dryness (yield = 12 mg dry weight per milliliter filtrate). The lyophilized filtrate was suspended in approximately 5% of the original filtrate volume (254 mg lyophilized filtrate/ ml) in ultra pure deionized water. The sample was then transferred to a 28 ml test tube and 9 ml of HPLC grade acetone were added to the sample. Samples were stored at 4 °C for 2 hours to allow precipitation. A visible precipitate formed during the first minute after acetone addition. The supernatant was decanted and filtered through a 0.45 μM pore size PTFE filter (Gelman Acrodisc®) to remove any particulate matter. The acetone:water phase was then evaporated under nitrogen at room temperature to a volume of approximately 4 mL to remove the majority of acetone. Approximately 80% of the bioassay activity was recovered in the dried liquid fraction. The yield was approximately 7 μg partially purified material per mg dry 500 MW filtrate precipitated.

Semi-preparative HPLC purification. Reverse phase semi-preparative HPLC purification was performed on a Gilson HPLC equipped with a Whatman semi-preparative HPLC column. The column had a length of 480 mm, an outer diameter of 16 mm, and an inner diameter of 9.5 mm. The column was packed with Partisil® 10 ODS-3

with a 10 μm particle size. (Whatman), and a 23.62 mL void volume. The HPLC system was also equipped with a UV detector, and absorbance was monitored at 260 and 340 nm. These monitoring wavelengths were chosen based on maxima for absorbances observed for the acetone purified preparation. Sample obtained following the partial acetone purification procedure outlined above was loaded onto the column with a 1 mL sample loop. The column temperature was maintained at 8°C using a continuous flow water jacket around the column and a Fisher model 9001 isotemp digital circulator. Flowrate of eluent was 7 mL/min, with 100% water for 5 min and a linear gradient to 100% methanol at 35 min. Samples were collected at 1 minute intervals, pooled, stripped under nitrogen to remove residual methanol, frozen under liquid nitrogen, and lyophilized to dryness. Lyophilized samples were rehydrated in SGW medium or in deionized water. The sample rehydrated with deionized water was saved for further analysis by storage at -20 °C and the portion rehydrated with SGW medium was combined with *P. fluorescens* and assayed for CCl_4 transformation.

Preparation of process blank. A process blank was prepared by filtering 1L of SGW medium (pH 8.2) through 300 KDa and 1KDa Filtron® ultracassettes, and lyophilizing the filtrate to dryness, rehydration, acetone extraction, and separation and collection of fractions by the same reverse phase HPLC method used in the purification procedure developed for the secreted factor(s).

RESULTS

Reconstitution of fractionated CCl_4 transformation activity. Figure 3.1 illustrates CCl_4 transformation by washed cells of strain KC, 500 MW filtrate, and reconstitution of washed cells of strain KC and the filtrate fraction. By themselves, washed cells did not

carry out appreciable transformation. However, rapid and reliable transformation was obtained when the washed cells were reconstituted with either of the filtrate fractions, indicating that the secreted factor(s) required for transformation is small, with a nominal molecular weight of less than 500 daltons. It should also be noted, however, that in some cases (see, for example, Table 3.1), CCl_4 transformation activity was observed with the partially purified cell free supernatant. Tatara et al. [13] reported a pseudo-first-order rate constant of 0.03 ± 0.03 min^{-1} for the cell-free activity in 13 independently grown cultures. The high standard deviation reflects the fact that for a large number of experiments, there was minimal or no cell free transformation activity. An example of the rates of CCl_4 degradation that can be achieved by the cell-free activity are shown in Table 3.1, however, it must be stressed that this example is not an average, but rather a characterization of the activity on two of the relatively rare occasions in which significant cell-free activity was observed.

Initially, strain KC cells were used in reconstitution assays. However, resuspended cells of strain KC continued to produce additional units of supernatant transformation component(s) during the time frame of the assay. After finding that other cell types could substitute for strain KC, a bioassay was developed using *P. fluorescens* to track production of the secreted activity [12]. *P. fluorescens* cells were preferred for the bioassay because, unlike strain KC, they are incapable of generating additional secreted transformation activity during the time-frame of the assay.

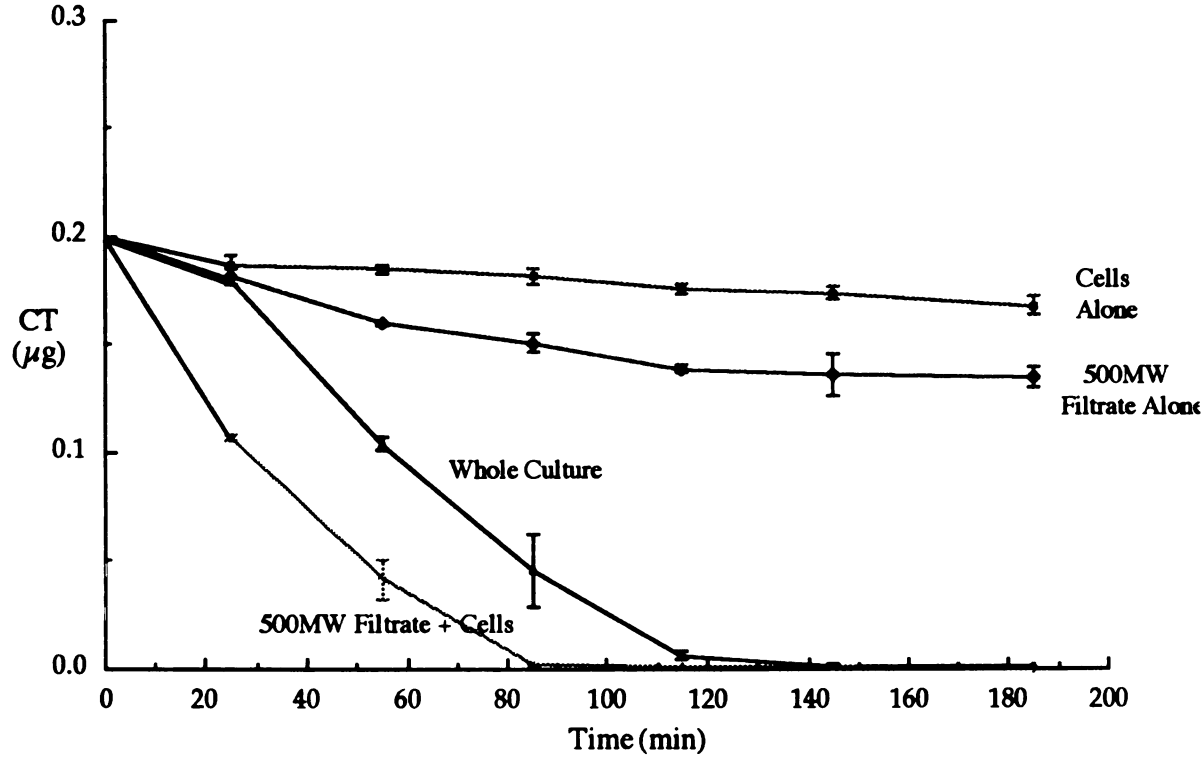

Figure 3.1. *P. stutzeri* KC cells combined with 500 MW filtrate from strain KC. *P. stutzeri* cells were grown for 24 hours in medium D (76 μg of protein per mL). Error bars represent the standard deviation of triplicate samples.

Table 3.1. Transformation kinetics for fractionated cell-free activity.

	Fraction	Pseudo second order rate coefficient [a] (L/mg protein-d)
Example 1	Complete culture	0.54[b]
	Supernatant	6.1
	10,000 MW Filtrate	21.3
Example 2	Complete culture	1.3
	500 mol weight filtrate	2.1

[a] Determined as per Tatara et al. (1993).

[b] Rate was calculated from the linear range of the transformation curve

Table 3.2. Iron binding properties of ultra-filtration fractions from culture supernatant produced by *P. stutzeri* KC.

	Measurements of iron binding activity		
Fraction	Rioux[a] (μM 2,3 DHBA)	C'sasky[b] (Δ abs @ 520 nm)	Neilands[c] (Δ abs @ 630 nm)
Blank	0	0	0
Supernatant	3.08	0.04	0.38
10 kD filtrate	2.18	0.02	0.39
0.5 - 10 kD retentate	3.27	0.59	0.41
<0.5 kD filtrate	1.57	0.04	0.34

[a] Presence of catechol quantified using 2,3-dihydroxybenzoic acid as standard

[b] Presence of hydroxymate was determined by measuring the absorbance at 520 nm. The concentration of hydroxymate was unable to be quantified by use of a hydroxylamine standard

[c]The level of iron binding activity was only tracked qualitatively.

Effects of iron and fractionation of iron binding activities. CCl_4 transformation by strain KC is inhibited by iron, and the possible role of a siderophore or siderophore-like agent has been previously proposed [1,12]. We sought to characterize the fractions generated during purification in terms of siderophore levels and sensitivity to iron inhibition. The results are shown in Table 3.2. The 500 MW filtrate exhibited substantial iron binding activity in the Neilands assay, accounting for approximately 50% of the total supernatant catechol class activity. Retentate above the 500 molecular weight filter exhibited iron binding activity in all three assays, indicating the production of multiple iron-binding activities by strain KC.

In marked contrast to the complete culture of *P. stutzeri* KC, 10,000 MW filtrate and 500 MW filtrate did not exhibit high iron sensitivity . Whole culture activity is inhibited by iron levels as low as 1 μM, but the combination of 10,000 MW filtrate and washed cells was not inhibited by 100 fold higher iron levels as shown in figure 3.2. This suggests that a high molecular weight secreted factor(s) (>10,000 MW) exacerbates or is responsible for the inhibitory effects of iron.

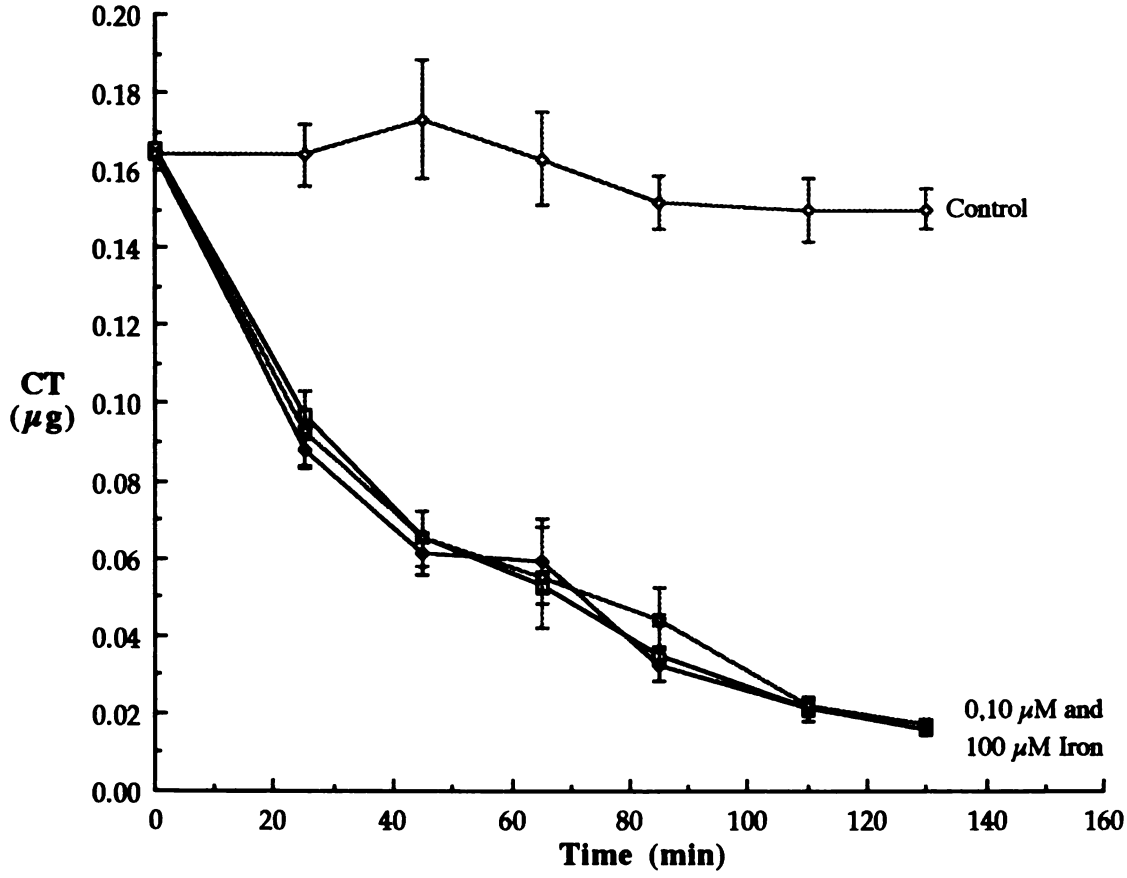

Figure 3.2. Effect on iron on transformation with 10,000 MW filtrate from strain KC. Error bars represent the standard deviation of triplicate samples.

Partial purification of secreted factor(s). Anion exchange perfusion chromatography revealed the presence of at least five constituents in the 500 MW filtrate. However, recovery of strong activity from a salt gradient elution was hindered by inhibition of transformation activity by ionic strength: 400 mM NaCl or KCl, as well as 200 mM Na_2PO_4 caused an approximate 50% inhibition of CCl_4 transformation activity. This salt concentration was in the center of the range in the salt gradient where elution of the major protein peaks occurred. Ammonium sulfate precipitation of the activity was also unsuccessful, possibly due to the observed effects of high ionic strength.

Acetone precipitation of concentrated lyophilized material was successful in removing the great majority of contaminating material, which rapidly precipitated at 90% acetone:10% water (v/v). Approximately 80% of the supernatant factor(s) (as measured in the *P. fluorescens* bioassay) remained soluble in the acetone:water phase, and was readily recovered by drying under nitrogen (Table 3.3).

Table 3.3. Results of acetone precipitation. All samples were assayed by reconstitution with *P. fluorescens* cells in SGW medium .

Fraction	**CCl_4 removed (μg)[a]**
abiotic control	0.001 ±0.001
positive control[b]	0.047 ±0.006
acetone supernatant	0.049 ±0.008
acetone pellet	0.013 ±0.004

a. ± one standard deviation
b. 10 mg of lyophilized 500 molecular weight filtrate

Semi Preparative HPLC Analysis. Semi-preparative HPLC analysis was performed to purify the secreted factor produced by *P. stutzeri* KC. Figure 3.3 illustrates a typical chromatogram for the initial HPLC separation of an acetone extract containing the secreted factor. Figure 3.4 illustrates a CCl_4 transformation activity plot of the fractions collected. The majority of CCl_4 transformation activity was recovered in fraction 28. Adjacent fractions also contained some CCl_4 transformation activity, but only fraction 28 was used for a second HPLC purification of the CCl_4 transforming factor. Fraction 28 from the secondary HPLC run did not contain any CCl_4 transformation activity, although it did contain an absorbance peak at 28 minutes. Fraction 28 from the primary and secondary rounds of HPLC purification were submitted for mass spectral analysis.

A process blank was prepared and analyzed by semi-preparative reverse phase HPLC to determine if any contaminants introduced from the purification process co-elute with the secreted factor. Figure 3.5 shows a chromatogram for initial HPLC separation of a sample containing the secreted factor versus a chromatogram of the process blank. A peak was present in the process blank at the same retention time as the CCl_4 transforming activity. Fraction 28 from the primary and secondary rounds of HPLC purification of the process blank were also submitted for mass spectral analysis.

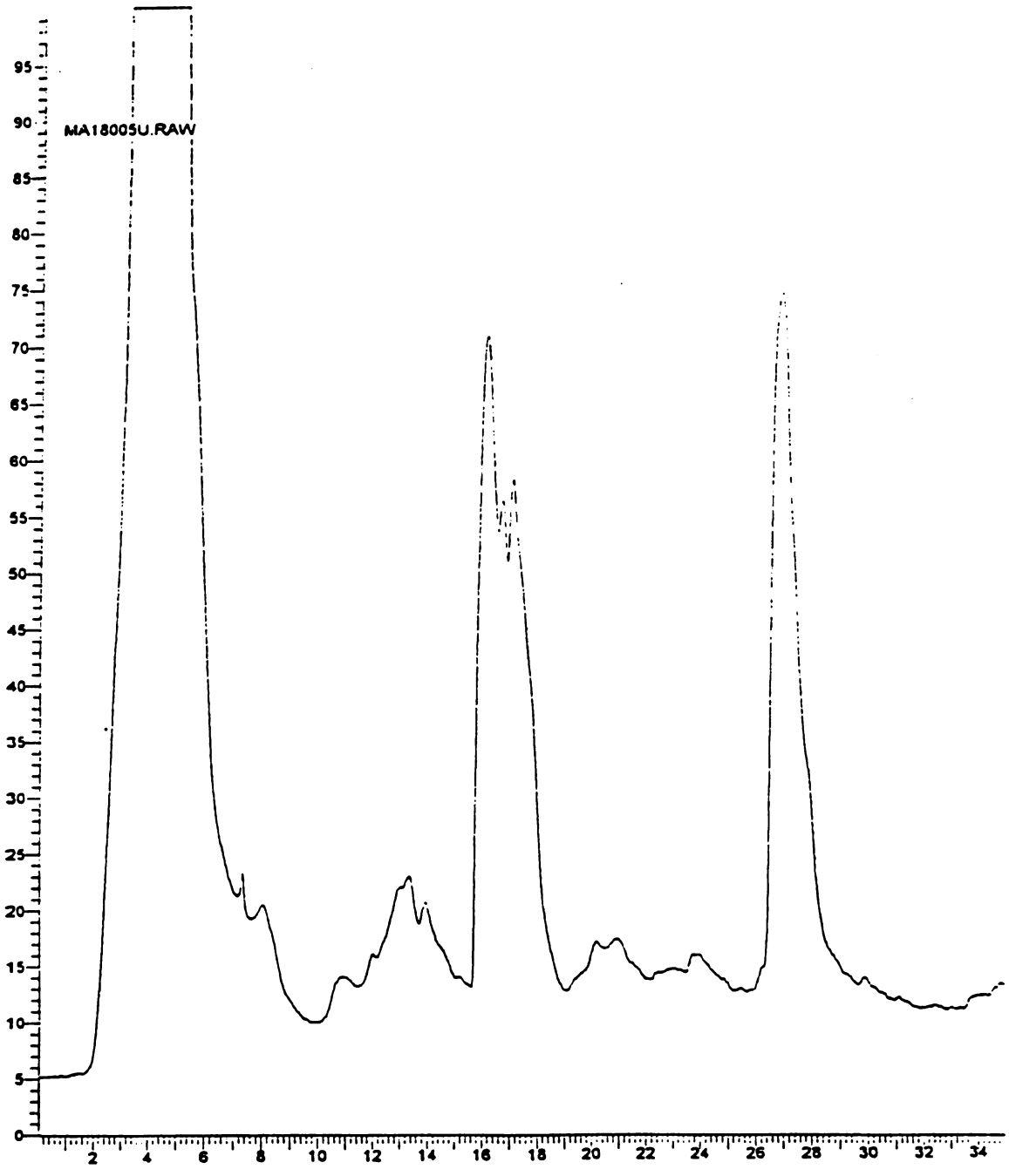

Figure 3.3. Semi-preparative HPLC chromatogram of a primary run of a sample containing the secreted factor involved in CCl_4 transformation. Sample absorbance was monitored at 260 nm.

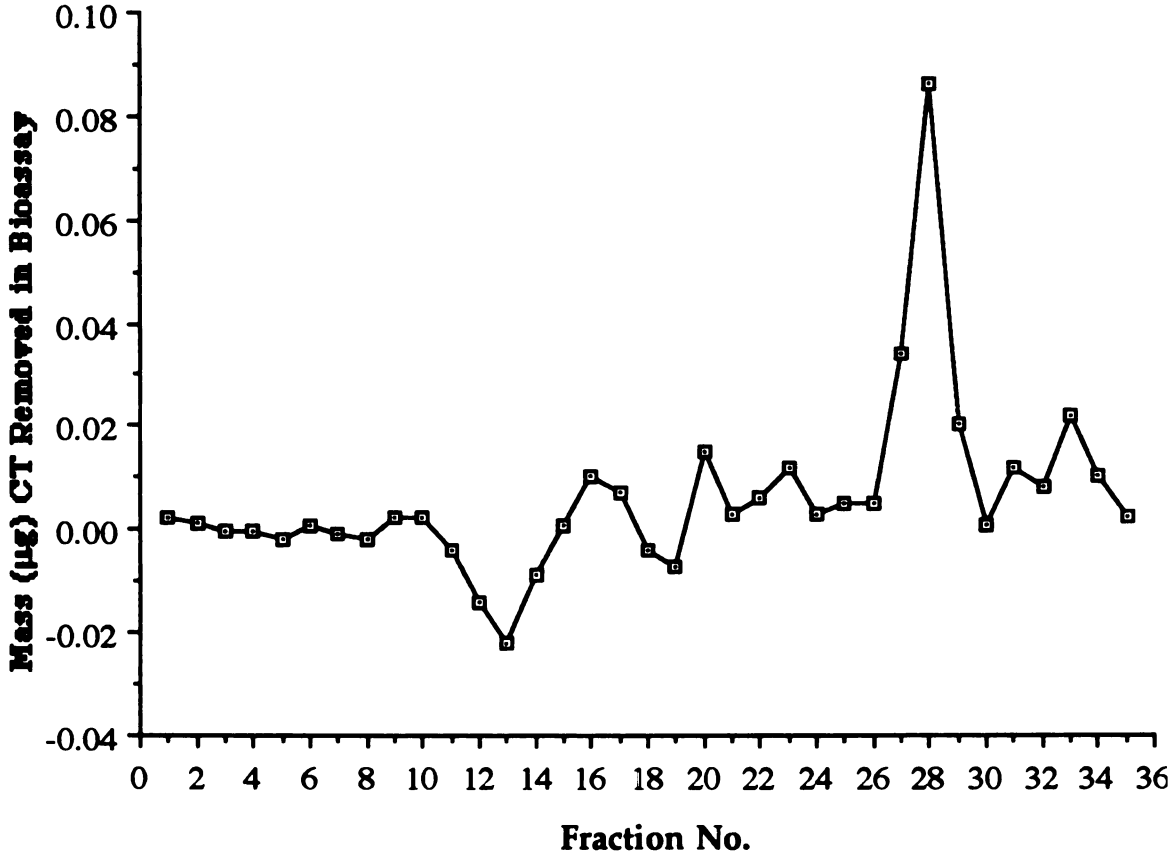

Figure 3.4. Mass of CCl_4 removed by fractions combined in a *P. fluorescens* bioassay following HPLC purification of acetone extracted CCl_4 transformation activity.

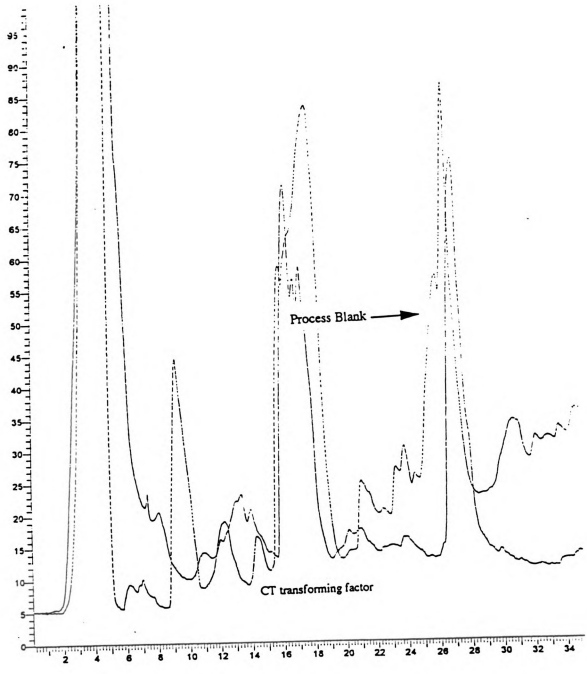

Figure 3.5. A semi-preparative HPLC chromatogram illustrating the initial separation of CCl_4 transforming activity. A chromatogram of the process blank is provided for comparison.

DISCUSSION

The underlying hypothesis evaluated in these studies was that an iron scavenging agent, more precisely a siderophore, was responsible for the fortuitous transformation of CCl_4 by *P. stutzeri* KC. The basis for this hypothesis was the inhibition of CCl_4 transformation by trace-levels of iron. Initially, the results presented in this chapter agreed with the iron scavenging hypothesis. The small size (< 500 daltons) of the secreted factor(s) was in the range of known iron binding siderophores, with reported molecular-masses between 360 to 1500 daltons [8]. Additionally, the occasionally observed cell-independent transformation activity suggested that under certain conditions, the transformation reaction is mediated by the secreted factor(s). However, the production of cell free activity by *P. stutzeri* KC was rare and was not reproducible, despite exhaustive efforts to understand its regulation. Consequently, further elucidation of the mechanism of CCl_4 transformation focused on cell-dependent transformation.

Evidence against the siderophore hypothesis was obtained from the experiments investigating iron inhibition in culture supernatant subjected to ultrafiltration. The finding that iron inhibition of CCl_4 transformation was alleviated when the supernatant was filtered through a 10,000 MW filter suggests that sensitivity to iron is conferred by a high-molecular-mass factor(s). This high-molecular-mass factor(s) may participate in iron-scavenging activities. The insensitivity of the <10,000 MW filtrate to iron inhibition suggests that the small secreted CCl_4 transforming factor(s) either does not play a direct role in iron binding or is still able to transform CCl_4 after binding iron. A possible explanation for these observations is that the normal physiological role of the low-molecular-mass CCl_4-transforming factor(s) is to shuttle electrons to a high-molecular-mass factor(s) that binds iron. When iron and the high-molecular-mass factor(s) are both present, electrons are transferred preferentially to the iron-binding factor(s) rather than

CCl_4. Such interpretation should be taken with caution, as further evidence is required to confirm or refute this hypothesis. In future experiments, the role of iron might be ascertained by monitoring reduction of Fe(III) to Fe(II).

Criddle et al. [1], Lewis and Crawford [5], and Tatara et al [12] have previously determined that induction of CCl_4 transformation occurs in iron-limited and copper-containing medium. It has also been suggested that electron transfer from the secreted factor to the CCl_4 molecule occurs [4,5,6]. Recently, Lewis and Crawford [6] were able to trap phosgene and thiophosgene as intermediates of CCl_4 transformation. These compounds are formed after an initial one-electron reduction of CCl_4. Dybas et al [4], recently showed that formate is an end-product of CCl_4 transformation, which requires a two net electron transfer onto the CCl_4 molecule. In Chapter 2, it was demonstrated that production of CO_2 and non-volatile products occurs simultaneously, with no interconversion of the products. These observations taken together with the results presented in this chapter suggests that a plausible model to explain the physiology of CCl_4 transformation may involve: (1) production and export of a CCl_4-transforming factor(s) from the cell in response to growth under iron-limitation and copper containing media, (2) deactivation or loss of electrons from the factor(s) upon transformation of CCl_4, and (3) reactivation or reduction of the factor(s) at the cell membrane. The question of whether a one- or two-electron reduction is involved requires additional investigation. The final elucidation of the CCl_4 transformation mechanism may depend on elucidation of the structure of the secreted factor involved in CCl_4 transformation.

An advance in the purification of the secreted factor(s) was achieved with the finding that CCl_4 transformation activity partitioned into the liquid phase following acetone precipitation. The partitioning of the secreted factor(s) into the acetone:water phase indicated that it may be slightly hydrophobic in nature. This finding was used to develop

a technique using reverse-phase HPLC to further purify the secreted factor(s). As shown in Figure 3.4, the majority of CCl_4 transformation activity is found in fraction 28. However, some activity was found in the adjacent fractions as well. This may be due in part to the manner in which fractions were collected from the HPLC and then pooled together for freeze drying. It is possible that the timing of collection for all fractions was not precise, and therefore some crossing over of fractions could have occurred during the collection process. Analysis of the process blank revealed that a contaminant peak also eluted at a retention time of 27-28 minutes during HPLC analysis (Figure 3.5). In the preliminary mass spectral analysis, common peaks were observed between both the process blank and the active sample, with no significant difference immediately apparent.

The purification procedure clearly needs additional development. Future HPLC runs should be monitored at more than just the 260 and 340 nm wavelengths. For example, absorbance maxima for polyenes are at 214 nm, aromatic rings at 253 nm and a, B unsaturated ketones at 215 nm [11]. Additionally, substituent corrections exist for each of these parent compounds [11]. Thus, knowing absorbance maxima may provided some structural information as to nature of the secreted factor(s). Loss of activity following the second-round of HPLC purification should also be examined. The nature of the secreted factor(s) could have changed following HPLC analysis, and eluted at a different time. Therefore, all fractions of the second purification should be combined with *P. fluorescens*, rather than only examining the fractions containing activity in the first round of HPLC purification. Finally, CCl_4 transformation activity may require more than one component. Thus, if a second HPLC purification run separates these components, activity will not be observed. In future investigations, all fractions should be pooled together and assayed for CCl_4 transformation.

In the following chapter, I describe studies which utilize the bioassay with *P.fluorescens* to examine properties of the CCl_4 transforming factor(s) as well as the cell types that can be combines with this factor(s) to result in CCl_4 transformation.

REFERENCES

1. **Criddle, C.S., J.T. DeWitt, D. Grbrić-Galić, and P.L. McCarty**. 1990. Transformation of carbon tetrachloride by *Pseudomonas* sp. strain KC under denitrifying conditions. *Appl. Environ. Microbiol.* **56**:3240-3246.

2. **Csa'ky, T.Z.** 1948. On the estimation of bound hydroxylamine in biological materials. *Acta Chem. Scand.* **2**: 450-454.

3. **Dybas, M.J. and J. Konisky.** 1989. Transport of coenzyme M (2-mercaptoethanesulfonic acid) and methylcoenzyme M [(2-methylthio)ethanesulfonic acid] in *Methanococcus voltae* : Identification of specific and general uptake systems. *J. Bact*. **171**:5866-5871.

4. **Dybas, M.J., G.T. Tatara, and C.S. Criddle.** 1995. Localization and characterization of the carbon tetrachloride transformation activity of Pseudomonas sp. strain KC. *Appl. Environ. Microbiol.* **61**:758-763.

5. **Lewis, T. A., and R. L. Crawford**. 1993. Physiological factors affecting carbon tetrachloride dehalogenation by the denitrifying bacterium *Pseudomonas* sp. strain KC. *Appl. Environ. Microbiol.* **59**:1635-1641.

6. **Lewis, T. A., and R. L. Crawford**. 1995. Transformation of carbon tetrachloride via sulfur and oxygen substitution by *Pseudomonas* sp. strain KC. *J. Bacteriol.* **177**: 2204-2208.

7. **Markwell, M.A., S. M. Haas, N.E. Tolbert, and L.L. Bieber**. 1981. Protein determination in lipoprotein samples: manual and automated procedures. *Methods Enzymol.* **72**:296-301.

8. **Neilands, J.B.** 1981. Microbial iron compounds. *Ann. Rev. Biochem.* 50: 715-731.

9. **Rioux, C., D.C. Jordan, and J.B.M. Rattray.** 1983. Colorometric determination of catechol siderophores in microbial cultures. *Anal. Biochem.* **133:** 163-169.

10. **Schwyn, B. and J.B. Neilands.** 1987. Universal chemical assay for the detection and determination of siderophores. *Anal. Biochem*. **160:** 47-56.

11. **Streitwieser, A. and C.H. Clayton**. 1981. Introduction to organic chemistry. 2nd. ed. MacMillan Publishing Co. New York.

12. **Tatara, G.M., M.J. Dybas, and C.S. Criddle**. 1993. Effects of medium and trace metals on kinetics of carbon tetrachloride transformation by *Pseudomonas* sp. strain KC. *Appl. Environ. Microbiol.* **59**:2126-2131.

13. **Tatara, G.M., M.J. Dybas, and C.S. Criddle.** 1995. Biofactor-mediated transformation of carbon tetrachloride by diverse cell types, p. 69-77. *In* R. Hinchee, A. Leeson, and L. Semprini (ed.) The Bioremediaton Series, **3(3)**, Bioremediation of Chlorinated Solvents. Battelle Press, Richland, WA.

CHAPTER 4

BIOFACTOR-MEDIATED TRANSFORMATION OF CARBON TETRACHLORIDE BY DIVERSE CELL TYPES

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ABSTRACT

The transformation of CCl_4 by *Pseudomonas stutzeri* KC requires a small (<500 dalton) factor(s) secreted by strain KC and cells capable of activating the factor. Partially purified supernatant from a culture of strain KC was combined with cells that do not transform CCl_4 or do so slowly. Rapid CCl_4 transformation was obtained using related *Pseudomonads* (*P. stutzeri* ; *P. fluorescens*); *Escherichia coli* K-12; a gram positive bacterium (*Bacillus subtilus*); a bacterial consortium derived from groundwater at Schoolcraft, MI (SC-1); a bacterial consortium derived from groundwater at Hanford, WA (HC-14); and yeast (*Saccharomyces cerevisiae*). Thus, specific cell types are not required for activation of the factor. This finding was used to develop a bioassay for the factor(s) in which samples to be assayed are inoculated with *P. fluorescens* , spiked with CCl_4, and monitored for CCl_4 degradation under anoxic incubation conditions. The bioassay was used to establish that aerobically-grown strain KC cells secrete the factor, oxygen reversibly inhibits CCl_4 transformation by the factor, live cells are needed for activation of the factor, the pH optimum for CCl_4 transformation is 8.5, the factor is readily transported through aquifer material, the factor is stable for up to six days in synthetic groundwater at 16°C, and the factor is stable indefinitely after lyophilization to powder and storage at -20°C. The possibility that organisms indigenous to aquifer solids can recharge the factor was evaluated by addition of the factor to a slurry of Hanford aquifer material. CCl_4 transformation was only obtained when aquifer material was inoculated with the secreted factor and biostimulated by addition of acetate and nitrate.

INTRODUCTION

The most significant strategies for *in situ* bioremediation are biostimulation, the addition of nutrients and/or substrates to stimulate indigenous populations, and bioaugmentation, the addition of non-indigenous organisms. Biostimulation avoids the problems associated with transport and survival of introduced organisms because the indigenous organisms are already present, and adapted to site conditions. A disadvantage of biostimulation is that some or all of the stimulated organisms may be unable to degrade the target contaminants, or, if they do degrade it, they may create undesirable products. The principal advantage of bioaugmentation is that the introduced organism and the transformation mediated by the organism, including the kinetics and pathway of transformation, can be studied and optimized under laboratory conditions. However, bioaugmentation also faces numerous challenges: the introduced organisms may fail to be transported, they may fail to colonize, or they may be unable to compete with the indigenous organisms. The ideal *in situ* remediation scheme would provide the benefits of bioaugmentation (pathway control and kinetic optimization) with the advantages of biostimulation (use of indigenous organisms).

Under denitrifying conditions, transformation of CCl_4 typically results in the slow accumulation of chloroform [4,7,13], a compound that is persistent and potentially harmful to human health [14]. As a result, remediation strategies that avoid chloroform production are desirable. *P. stutzeri* KC rapidly transforms CCl_4 to carbon dioxide [5,11,15], formate [6], and unidentified non-volatile product(s), without the production of chloroform [5,15]. Thus, use of this transformation system offers pathway and kinetic advantages. As shown by Dybas et al. [6], strain KC produces a small (< 500 dalton) secreted factor that is required for CCl_4 transformation. This secreted factor is regenerated in the presence of viable whole cells of *P. stutzeri* KC.

In this chapter, evidence is provided that a diverse range of microorganisms regenerate CCl_4 transformation activity, including organisms that are indigenous to CCl_4-contaminated aquifers. Several characteristics of the factor activity are evaluated including stability of the secreted factor, its production under aerobic conditions, oxygen inhibition of CCl_4 transformation, and transport of the factor through aquifer material. While others have observed secreted xenobiotic degradative activities [2,8], this appears to be the first report of a non-specific biological process for regeneration of extracellular degradative activity.

MATERIALS AND METHODS

Chemicals. CCl_4 (99% purity) was obtained from Aldrich Chemical Co., Milwaukee, Wis. All chemicals used were ACS reagent grade (Aldrich or Sigma Chemical Co.). All water used in reagent preparation was deionized 18 Mohm resistance or greater.

Media and growth conditions. Medium D prepared as described in Chapter 2. Simulated groundwater medium (SGW) was prepared as described in Chapter 3.

Yeast medium contained per liter of deionized water: 75 g of glucose, 3 beef bouillon cubes, 2 g of KH_2PO_4 and 3 g of K_2HPO_4. The pH of the yeast medium was adjusted to 8.0 with 3 N KOH and autoclaved at 121°C for 20 minutes prior to use.

P. stutzeri KC (ATCC deposit no. 55595, DSM deposit no. 7136) was grown in medium D or SGW medium under an atmosphere of nitrogen in modified 500 mL Wheaton bottles (Wheaton cat. no. 219819). Openings to permit sampling were created in the bottle caps by drilling 12 mm holes through the caps and inserting 30 mm teflon-faced butyl rubber septa (Wheaton no. 224174). Sealed bottles were pressure tested for leakage at 5 psi.

All cultures of strain KC were shaken at 150-200 rpm on a rotary shaker at a temperature of 20 - 23°C. Culture manipulations were typically performed in the anaerobic glove box under an atmosphere of 97±2% N_2 and 3±2% H_2. Oxygen and hydrogen levels were monitored continuously with a Coy model 10 gas analyzer.

Analytical methods. CCl_4 transformation assays were performed as described in Chapter 2. The procedure described in chapter 2 was also used to evaluate the possibility

of chloroform production. In all assays, the method detection limit was 0.01 μg for chloroform, corresponding to an aqueous phase concentration of 2 μg/L, as determined by Standard Methods 1030E [1].

Preparation of partially purified culture supernatant. Cultures and supernatant were generally prepared and manipulated in the anaerobic glove box. Cultures of strain KC were screened for CCl_4 transformation activity prior to the preparation of partially purified culture supernatant. Actively transforming culture was transferred to 40-mL Nalgene® centrifuge tubes and centrifuged at 4°C and 27,200 x g. Centrifuged supernatant was decanted into a 115-mL sterile 0.2 μm filter unit, and a hand vacuum pump was used to filter out any remaining cells from the culture supernatant. The resulting 0.2 μm filtrate was loaded into a model 8400 Amicon ultrafiltration stirred cell and filtered at 30 psi through a preconditioned PM 10 (10,000 molecular weight cut-off) ultrafiltration membrane (Amicon no. 13142). The 10,000 MW (molecular weight) filtrate obtained in this manner was either used directly in CCl_4 transformation assays or further filtered at 55 psi through a preconditioned YC 05 (500 MW cut-off) ultrafiltration membrane (Amicon no. 13042). 500 MW filtrate was collected in a sterile serum bottle on ice.

Experiments with diverse cell types. *Pseudomonas fluorescens* (ATCC deposit no. 13525), *Escherichia coli* K-12 (ATCC deposit no. 10798), and *Bacillus subtilis* (ATCC deposit no. 6051) were obtained from the culture collection of the Microbiology Department at Michigan State University. *Pseudomonas stutzeri* strain EP3-071388 11G, an aquifer isolate from Seal Beach, Calif., was provided by H. Ridgway (Orange County Water District, CA). An aquifer consortium, designated SC-1, was obtained by enrichment of organisms in a groundwater sample from a CCl_4-contaminated aquifer at Schoolcraft, MI. The enrichment was obtained from a 1% inoculum of Schoolcraft

groundwater in Nutrient Broth (Difco, Co.). A second aquifer consortium, designated HC-14, was obtained from R. Skeen of Battelle Pacific Northwest Laboratories (Richland, WA). HC-14 was derived from a sample of aquifer material at the Hanford, WA site. *Saccharomyces cerevisiae* (ATCC #58527) was obtained from Red Star Co., Milwaukee, WI.

P. fluorescens , *P. stutzeri* , and SC-1 were grown at 20-23°C under denitrifying conditions in medium D (pH 8.2) or aerobically in medium D (pH 7.0) supplemented with 10 μM $FeSO_4$. *B. subtilis* and in some instances *P. fluorescens* were grown at 20-23°C aerobically in nutrient broth (Difco Co.). *E. coli* was grown at 35°C aerobically in medium D, but with glucose (3 g/L) instead of acetate as the electron donor. HC-14 was grown under denitrifying conditions in SGW medium (pH 7.5). *S. cerevisiae* was grown aerobically at 35°C in yeast medium.

Cultures were transferred to an anaerobic glove box and dispensed into 40-mL centrifuge tubes. Cells were collected by centrifuging at 12,100 x g for 5 minutes , wasting the culture supernatant, and resuspending the pellet in 4 mL of anoxic medium D or anoxic medium SGW at pH 8 or pH 7.5. Cultures of *E. coli* or *B. subtilis* were resuspended in anoxic medium D with glucose as the carbon source, and *S. cerevisiae* cultures were resuspended in anoxic yeast medium. A 0.5 mL sample of cell suspension (concentrated ten fold by centrifugation) was added to 4.5 mL of filtered supernatant and assayed for CCl_4 transformation in 28-mL aluminum seal tubes as described previously.

Growth of *P. stuzeri* KC and secreted factor(s) production. Cultures of *P. stutzeri* KC were grown under denitrifying conditions in SGW medium. Cultures were inoculated with a 0.5% (v/v) inoculum taken from a 96 hour starter culture of strain KC grown in SGW medium. Cultures were grown in 500 mL Wheaton bottles as previously described.

Initial and subsequent samples were taken by withdrawing 6mL of liquid, freezing 1 mL for protein analysis, and filtering 5 mL of sample through a 0.22 μm nylon filter. The 5 mL filtered sample was then frozen and lyophilized. Following completion of the growth study, which was terminated at 100 hours following inoculation, the lyophilized samples were rehydrated to 5 mL with deionized water and assayed for secreted factor using the *P. fluorescens* bioassay.

Aerobic production of the supernatant factor. Cultures of strain KC were grown aerobically in medium D. In the late exponential phase, cultures were filtered through a 0.2 μm filter. The resulting filtrate was made anoxic by transfer through the interlock of an anaerobic glove box, filtered through a 10,000 MW filter, then filtered again through a 500 MW filter. A portion of the 500 MW filtrate was dispensed anaerobically as 4.5 mL aliquots into 28 mL serum tubes, spiked with 0.5 mL of an anoxic cell suspension of *P. fluorescens*, and assayed for CCl_4 transformation. The remainder was removed from the glove box, aerated, dispensed as 5 mL aliquots into 28 mL serum tubes (air headspace), spiked with *P. fluorescens* cells, and assayed for CCl_4 transformation.

Slurry experiments with aquifer material. Approximately 2 g (wet weight) of Hanford aquifer solids (provided by R. Skeen, Battelle Pacific Northwest Laboratories, Richland, WA) were placed into sterile 28-mL aluminum seal tubes. Each tube received 2 mL of SGW medium (pH 8.2) containing 800 mg/L of NO_3^-. One set of tubes was autoclaved (sterile controls), one was amended with 800 mg/L acetate, and one received no amendments. All tubes were spiked with 0.5 μg CCl_4 and shaken at 20°C. After three days, the acetate-fed tubes received an additional spike of 800 mg/L acetate and 800 mg/L nitrate. Two days later, half of the tubes from each set were supplemented with 2 mL of 10,000 MW filtrate from a culture of strain KC grown in SGW medium under denitrifying conditions. Tubes that did not receive the filtrate were spiked with 2 mL of

anoxic SGW medium at pH 8.2. All tubes were incubated for 48 hours at 20°C and heated at 70°C for two hours to release sorbed CCl_4. Gas phase CCl_4 mass was determined using equivalent volume aqueous phase standards (prepared as previously described) that were also heated to 70°C.

Stability of CCl_4 transformation in the supernatant fractions. The stability of the CCl_4 transforming factor was evaluated under different storage conditions. In one test, 500 MW filtrate obtained from a culture of strain KC grown in medium D (pH 8.2) was stored at 0°C under nitrogen in a 160-mL serum bottle sealed with teflon-lined butyl rubber septum. Five milliliter factions were removed daily, combined with cells of *P. fluorescens* , and assayed for CCl_4 transformation. In a second test, 10,000 MW filtrate from a culture of strain KC grown in SGW medium at pH 8.2 (pH 8.3 after growth) was divided into two fractions, and the pH of one fraction was adjusted to 7.5. Both fractions were filter sterilized through a 0.2 μm filter, sealed under a headspace of nitrogen, and stored at 16°C in a 160-mL serum bottle sealed with a teflon-lined butyl rubber septum. Five milliliter fractions were removed daily, combined with cells of *P. fluorescens,* and assayed for CCl_4 transformation.

CCl_4 transformation using lyophilized culture filtrate. 10,000 MW filtrate from strain KC grown in SGW medium was frozen overnight at -20°C and lyophilized. The resulting powder was stored at -20°C under aerobic conditions. Freeze-dried powder was rehydrated in deionized water at one- or two-times its original concentration. Rehydrated powder was then deoxygenated by transfer through the interlock of an anaerobic glove box, dispensed into 28 mL aluminum seal tubes, mixed with *P. fluorescens* , spiked with CCl_4, and assayed for CCl_4 transformation. To obtain the transformation capacity of freeze-dried powder, samples were respiked with CCl_4 until CCl_4 transformation stopped.

Requirement for live cells. Cultures of *P. fluorescens* were grown aerobically in Nutrient Broth (Difco. Co.). The cultures were split into two equal fractions, one of which was autoclaved at 121°C for 15 minutes. After autoclaving, the cultures were confirmed killed by plating 100 μL of autoclaved culture onto nutrient agar (Difco Co.) plates and incubating at 20°C under aerobic conditions for 5 days. Both the autoclaved and live cultures of *P. fluorescens* cultures were transferred to an anaerobic glove box and dispensed into sterile 40-mL centrifuge tubes. Cells were collected by centrifugation at 12,100 x g, and pellets were resuspended in 4 mL of SGW medium at pH 8. A 0.5 mL sample of cell suspension was added to 4.5 mL of rehydrated 10,000 MW filtrate and assayed for CCl_4 transformation in 28-mL tubes as described previously.

Transformation rate dependence on cell density. Freeze dried 10,000 MW filtrate from *P. stutzeri* KC was prepared as previously described. *P. fluorescens* was grown 24 hours in SGW medium under aerobic conditions from a 1% nutrient broth inoculum. Eighty milliliters of *P. fluorescens* was centrifuged at 10,000 rpm for 5 min. in a SS-34 rotor and concentrated 10X by resuspending the pellet in 8 mL of fresh SGW medium. Various quantities of the resulting cell concentrate were added to tubes containing 5 mL of 10,000 MW filtrate that had been rehydrated at its original concentration from freeze dried powder. The cell concentration range was from zero to 1.4×10^8 CFU/mL. Cell concentration was determined by plating a dilution series of culture concentrate on nutrient agar and counting colony forming units per milliliter.

pH optimum for CCl_4 transformation. Freeze dried 10,000 MW filtrate from *P. stutzeri* KC was prepared as previously described. Following rehydration, the filtrate was dispensed into 20 mL aliquots, and the pH of individual aliquots was adjusted with 1 M HCl or with 1 M NaOH to cover the pH range from 6 through 10 in pH increments of 0.5.

Three *P. fluorescens* cultures were grown 24 - 36 hours in SGW medium at pH 6.5, 7.5, and 8.5 under aerobic conditions from a 1% nutrient broth inoculum. Eighty milliliters from each culture were centrifuged and resuspended in 9 mL of phosphate buffer at the desired pH level. The culture grown at pH 6.5 was used to test optimum at pH 6.0 and 6.5. The culture grown at pH 7.5 was used to test the pH optimum at pH 7.0, 7.5 and 8.0. The culture grown at pH 8.5 was used to test the pH optimum at 8.5, 9.0 and 10.0. Cells were combined with the pH adjusted filtrate, and CCl_4 transformation activity was measured as previously described. Final pH readings were recorded and graphed against the first order rate coefficient for CCl_4 transformation (k").

Transport of the secreted factor through aquifer material. Kontes® glass columns (30 cm length, 2 cm i. d.) fitted with Teflon® luer lock stopcocks were sanitized by soaking in a solution of 0.06% hypochlorite, rinsed with sterile deionized water, and packed aseptically with Hanford or Schoolcraft aquifer material under a laminar hood. To create a slurry for packing, Hanford aquifer material (100 g wet weight) was combined with SGW medium containing 400 mg/L nitrate. Fines were removed by repeated elutriation with SGW medium, and large particles (>8 mm) were also removed. These materials were removed to enable flow to pass through the solids at a reasonable rate (2.5 mL/min) without creating a large back pressure. To create a slurry, Schoolcraft aquifer material (courtesy of Brown & Root Environmental, Holt, MI) was combined with SGW containing 400 mg/L NO_3^-. In this case, adequate flow was achieved without removing fines or large particles. Slurries of Hanford and Schoolcraft aquifer material were poured directly into columns containing SGW medium and periodically tapped during filling to provide uniform packing and prevent the entrainment of air pockets. After packing, each column received three exchanges of SGW medium to remove small bubbles and to stabilize the packing. Each column was then connected to a Harvard® syringe pump, and flow of SGW medium containing 10 μCi of ^3H_2O per liter was

initiated at 2.5 mL/min. Breakthrough was monitored by collecting 5 mL fractions of column effluent and measuring the radioactivity in each fraction. Samples of each fraction (300 μL) were injected into 10 mL of Safety Solve® (Research Products International Corporation) liquid scintillation fluor and counted on a Model 1500 Packard Tri Carb liquid scintillation counter. The porosity, as estimated from the breakthrough curves, was 0.48 for the Hanford packed column and 0.46 for the Schoolcraft packed column.

Mobility of the secreted factor(s) was evaluated by pumping 10,000 MW filtrate from strain KC grown in SGW medium through both columns at a rate of 2.5 mL/min. Effluent from each column was collected in 5 mL fractions and assayed for secreted factor using the *P. fluorescens* bioassay.

Modeling. Tatara et al. [16] demonstrated that CCl_4 transformation by strain KC is first order with respect to CCl_4 concentration over the CCl_4 concentration range used in this study. Assuming a first order kinetic expression and equilibrium between the gas and liquid phase, the following equation can be written:

$$\frac{-dM_{CT}}{dt} = \frac{k''}{V_{aq}+H_c V_g} M_{CT} V_{aq}$$

where M_{CCl_4} is the micrograms of CCl_4 in the bottle, t is time in minutes, k" is the apparent first order rate coefficient (min^{-1}), V_{aq} is the aqueous phase volume, V_g is the gas phase volume, and H_C is the dimensionless Henry's constant for CCl_4 (1.0 at 20°C). Separation of variables and integration yields:

$$\ln\left(\frac{M_{CT}}{M^{o}_{CT}}\right) = -\left(\frac{k'' V_{aq}}{V_{aq}+H_c V_g}\right) t$$

For a reaction that is first order with respect to CCl_4 concentration, a plot of the logarithm of the mass of CCl_4 in the bottle versus time yields a straight line with slope = $\frac{-k'' V_{aq}}{V_{aq}+H_c V_g}$. Rearrangement and solving for k" yields: $k'' = -\text{slope}\left(\frac{V_{aq}+H_c V_g}{V_{aq}}\right)$. The half-life ($t_{1/2}$) is given by: $t_{1/2} = \frac{0.693}{k''}$.

RESULTS

Transformation of CCl_4 with diverse cell types. As illustrated in Figure 4.1, the partially purified supernatant transformed CCl_4 to a limited extent by itself, but its activity was highly variable, as indicated by the large standard deviation for this sample set in Table 4.1. In the presence of viable cells, the rate and reliability of transformation increased dramatically. Figure 4.1 shows a typical transformation pattern. By themselves, cells of *P. fluorescens* were unable to transform CCl_4, but when combined with 500 MW filtrate, rapid transformation resulted. Because *P. fluorescens* is unable to transform CCl_4 and does not produce any CCl_4-transforming secreted factor, the relative level of secreted factor in a sample can be determined by adding CCl_4 to *P. fluorescens* and monitoring the rate of CCl_4 transformation. This simple procedure was used in subsequent experiments as a bioassay for the secreted factor.

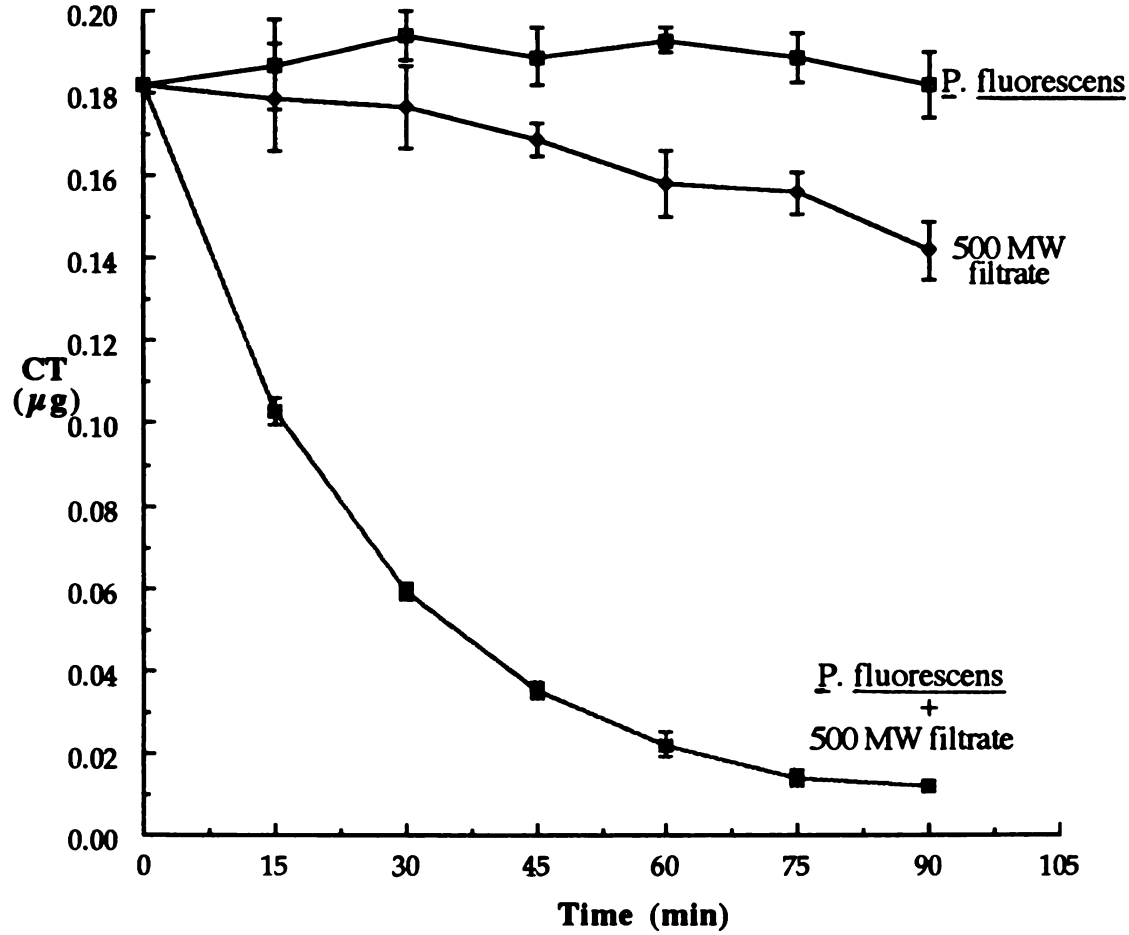

Figure 4.1. Rapid CCl_4 transformation results from the combination of *P. fluorescens* with 500 MW filtrate. Error bars represent the standard deviation of triplicate samples.

As shown in Table 4.1, CCl_4 transformation occurs when a wide range of cell types are incubated in the presence of the secreted factor produced by strain KC. CCl_4 transformation was obtained using cells from related *Pseudomonads* (*P. fluorescens*, *P. stutzeri*), another gram negative organism (*E. coli*); a gram positive organism (*B.subtilus*); a consortium of organisms enriched from CCl_4-contaminated groundwater from Schoolcraft, MI (SC-1); a consortium of organisms enriched from CCl_4-contaminated aquifer solids from Hanford, WA (HC-14); and yeast (*S. cerevisiae*). By itself, the Hanford enrichment was unable to transform appreciable CCl_4 within the time frame of this experiment, but when incubated in the presence of the secreted factor, a CCl_4 half-life of only 2.7 ± 0.2 minutes was obtained.

Table 4.1 First-order rates coefficients and half-lives of CCl_4 transformation .

Cell type	Growth conditions [a]	Secreted factor added?[b]	k'' (min^{-1}) [c]	CCl_4 half life (min)	n [d]
secreted factor alone (No cells present)	medium D, aerobic or anoxic	NA	0.03 ± 0.03	67 ± 65	13
P. stutzeri KC	medium D, anoxic	Yes	0.18 ± 0.05	4.0 ± 1.1	6
P. fluorescens	medium D, aerobic	No	0.00 ± 0.00	NA	3
		Yes	0.20 ± 0.01	3.4 ± 0.1	3
	nutrient broth	No	0.00 ± 0.00	NA	3
		Yes	0.10 ± 0.00	7.2 ± 0.2	3
P.stutzeri	medium D, aerobic	No	0.00 ± 0.00	NA	3
		Yes	0.15 ± 0.00	4.5 ± 0.0	3
E. coli K-12	med D, glucose, aerobic	No	0.00 ± 0.00	NA	3
		Yes	0.06 ± 0.01	12 ± 1	3
B. subtilus	nutrient broth	No	0.00 ± 0.00	NA	3
		Yes	0.04 ± 0.01	19 ± 3	3
Schoolcraft Consortium (SC-1)	medium D, aerobic	No	0.00 ± 0.00	NA	3
		Yes	0.25 ± 0.01	2.8 ± 0.1	3
	med. D, pH 7, 10 μM Fe, aerobic	No	0.00 ± 0.0	NA	3
		Yes	0.28 ± 0.03	2.5 ± 0.3	3
Hanford Consortium (HC-14)	SGW pH 7.5 anoxic	No	0.00 ± 0.00	NA	3
		Yes[e]	0.25 ± 0.02	2.7 ± 0.2	3
S. cerevisiae	yeast medium	No	0.00 ± 0.00	NA	3
		Yes[e]	0.12 ± 0.01	5.8 ± 0.5	3

[a] all cells were grown at pH 8.2 unless otherwise noted.

[b] secreted factor added as 500 MW filtrate unless otherwise noted.

[c] ± represents the standard deviation of the designated n number of samples

[d] n = number of samples.

[e] secreted factor added as 10,000 MW filtrate.

The single gram-positive organism evaluated in this study (*B. subtilus*) exhibited slower CCl_4 transformation upon combination with the supernatant factor than the gram negative organisms (Table 4.1). This may be related to differences in membrane or peptidoglycan structure, but additional confirmation is needed. The ability of a eukaryote (*S. cerevisiae*) to transform CCl_4 in the presence of the secreted factor produced by strain KC demonstrates that the ability to transform CCl_4 in the presence of the factor is very general and may be universal. Also of interest is the finding that cultures that are combined with the secreted factor do not need to be grown under iron-limiting conditions. Cultures of *P. fluorescens* and *B. subtilus* grown in nutrient broth and Schoolcraft consortium SC-1 grown at pH 7 with 10 μM iron were all able to transform CCl_4 in the presence of the secreted factor (Table 4.1). Thus, while production of the secreted factor by strain KC is dependent upon iron-limiting conditions [5,10,15], transformation in the presence of the factor is not.

Chloroform was not detected during CCl_4 transformation by any of the supernatant/cell combinations tested (chloroform method detection limit = 2 μg/L). This is consistent with previous reports of CCl_4 transformation products for strain KC [10,15].

Growth of *P. stuzeri* KC and secreted factor(s) production. The ability of samples to be stored and assayed for CCl_4 transformation using the *P. fluorescens* bioassay was used to determine the phase of growth during which *P. stutzeri* KC produces and secretes the CCl_4-transforming factor. Figure 4.2 illustrates the growth of *P. stutzeri* KC and biomolecule production versus time as measured by the first-order-rate coefficient for CCl_4 transformation in the bioassay. A very rapid rate of growth is observed at 6 hours following inoculation, during which time no biomolecule production is observed. After approximately 12 hours of growth, rapid production of biomolecule is observed until the

culture reaches an age of 36 hours. After this point, no significant increase in biomolecule production is observed.

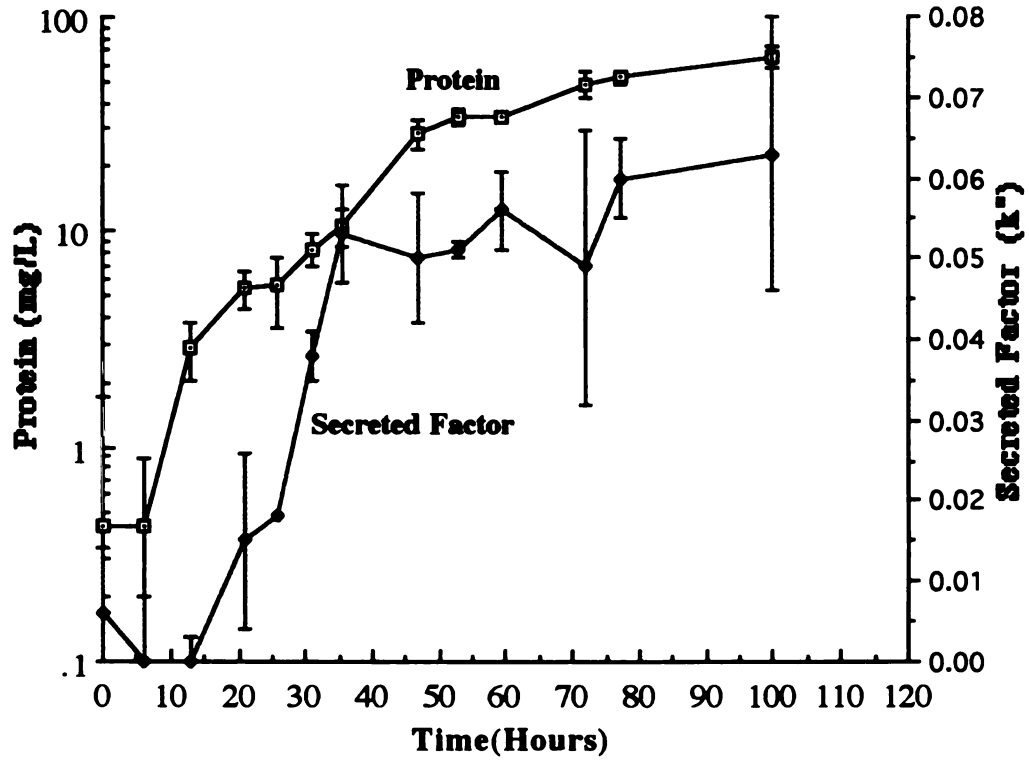

Figure 4.2. Secreted factor production during the growth of *P. stutzeri* KC. Error bars represent the standard deviation of three independently grown cultures.

Production of the secreted factor under aerobic conditions. The bioassay with *P. fluorescens* was used to determine whether secreted factor is produced by aerobically grown cells of strain KC. Filtered supernatant from an aerobically grown culture of strain KC was combined with cells of *P. fluorescens*. As shown in Figure 4.3, CCl_4 transformation occurred when the filtered supernatant or filtered supernatant/cell mixture was incubated under anoxic conditions, indicating that strain KC can and does produce

the factor aerobically. Production of the factor appears to be growth-associated, as higher biomass levels were obtained for aerobically grown cells. It is interesting to note, however, that CCl_4 transformation did not occur when the supernatant or supernatant/cell mixture was incubated aerobically, indicating that molecular oxygen or one of its products inhibits the transformation. It should also be noted that production of the secreted factor was inhibited by iron, regardless of the electron acceptor used for growth. No CCl_4-transforming secreted factor was produced when strain KC was grown aerobically in medium containing more than 10 μM iron (data not shown).

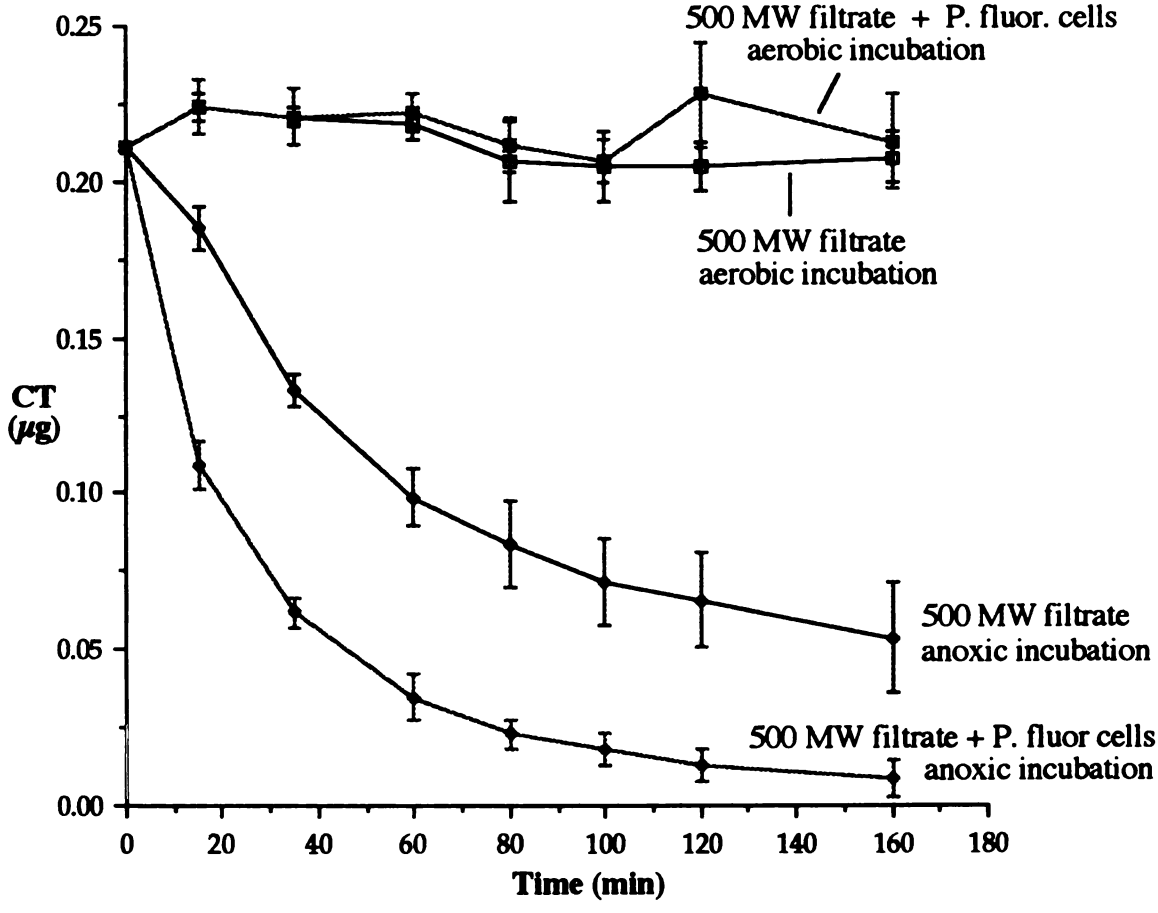

Figure 4.3. Incubation under aerobic conditions inhibits CCl_4 transformation. Error bars represent the standard deviation of triplicate samples.

Stability of the secreted factor. To assess the potential for application of the secreted factor, its stability was evaluated under various storage conditions. Initially, 500 MW filtrate from strain KC (grown in medium D) was stored under a headspace of N_2 at 0°C. Under these conditions, CCl_4 transformation activity was stable for 2 days but fell off on day 3 (Table 4.2). No activity was observed on day 5. Subsequently, stability of 10,000 MW filtrate from strain KC (grown in SGW medium) was evaluated when stored at 16°C under a headspace of nitrogen. Under these conditions, transformation activity persisted for four days, began to decrease after six days, and was undetectable by the seventh day. As shown in Table 4.2, initial transformation activity was significantly higher when CCl_4 transformation assays were performed at pH 8.3 than at pH 7.5, indicating that transformation kinetics are pH dependent.

Table 4.2. Stability of the secreted factor(s) as indicated by changes in the apparent first-order rate constants (k") under varied storage conditions. [a]

	Storage Conditions		
Time (days)	**Medium D, pH 8.2, 0°C, under nitrogen**	**SGW medium, pH 8.3, 16°C, under nitrogen**	**SGW medium, pH 7.5, 16°C, under nitrogen**
0	0.19 ± 0.01 [b]	0.25 ± 0.01	0.15 ± 0.01
1	0.25 ± 0.01	0.19 ± 0.02	0.06 ± 0.01
2	0.21 ± 0.01	0.20 ± 0.02	0.10 ± 0.04
3	0.02 [c]	0.15 ± 0.01	0.06 ± 0.01
4	ND [d]	0.15 ± 0.01	0.05 ± 0.00
5	0.00 ±0.00	ND	ND
6	ND	0.09 ± 0.03	0.02 ± 0.01
7	ND	0.00 ± 0.00	0.00 ± 0.00

[a] tabulated values represent the apparent first order rate constants [k" (min^{-1})], obtained by combining the stored supernatant with *P. fluorescens* cells and measuring the initial rate of CCl4 transformation.
[b] ± values represent the standard deviation of triplicate samples.
[c] no ± values are shown as this data point represents an average of duplicate samples
[d] ND = not determined.

To further assess the stability of the secreted factor and to provide a possible means of concentrating it, 10,000 MW filtrate from strain KC grown in SGW was frozen at -20°C and lyophilized to dryness. Lyophilized powder was stored at -20°C for six days prior to rehydration. The lyophilized powder was rehydrated at one- and two-times its original concentration, and then combined with cells of *P. fluorescens* . As shown in Table 4.3, significant CCl_4 transformation was observed. Combination of cells with powder rehydrated to twice its original concentration resulted in more extensive transformation than the combination of cells and powder rehydrated to its original concentration.

However, the increased mass of CCl_4 transformed was not proportional to the increase in powder concentration.

Table 4.3. Concentration dependence of the secreted factor on transformation of CCl_4 by *P. fluorescens* cells.

Test sample	**CCl_4 removed after a 24 hour incubation (μg)**
1) *P. fluorescens* alone	0.08±0.04
2) *P. fluorescens* + 1X rehydrated culture filtrate [a]	0.87±0.03
3) *P. fluorescens* + 2X rehydrated culture filtrate [b]	0.99±0.01

[a] 1X represents a freeze-dried powder prepared from 10,000 MW filtrate that was rehydrated to its original concentration.
[b] 2X represents a freeze-dried powder prepared from 10,000 MW filtrate that was rehydrated to twice its original concentration.

Requirement for live cells. The bioassay with *P. fluorescens* was used to determine whether live cells are required for CCl_4 transformation. Rehydrated culture filtrate was combined with live and dead (autoclaved) cells of *P. fluorescens* . As shown in Figure 4.4, CCl_4 was rapidly transformed when the 10,000 MW filtrate was combined with live cells, but transformation was not observed when filtrate was recombined with autoclaved cells. Thus, live cells are required for the transformation.

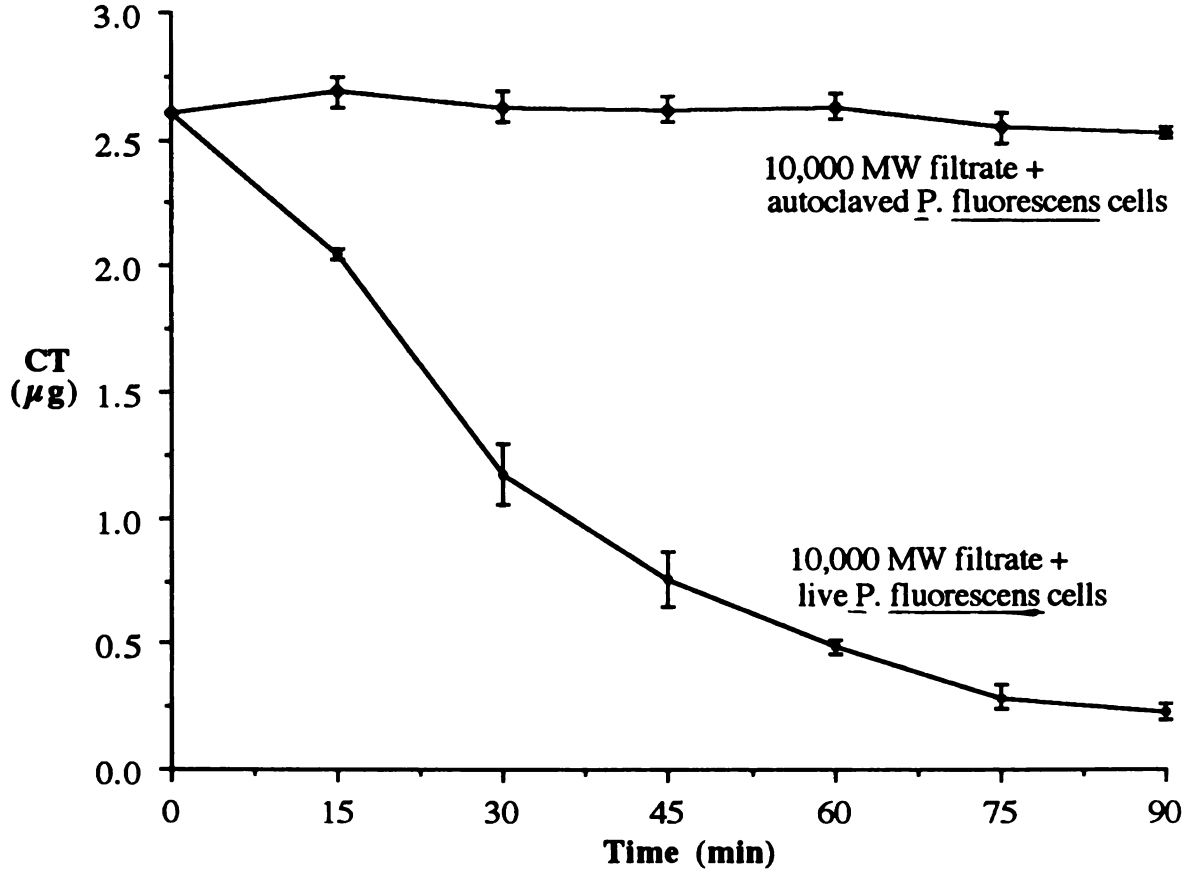

Figure 4.4. Effect of cell viability on CCl_4 transformation. Secreted factor was added as freeze dried 10,000 MW filtrate from strain KC grown in SGW medium. Error bars represent the standard deviation of triplicate samples.

Transformation rate dependence on cell density. The *P. fluorescens* bioassay was used to establish that CCl_4 transformation kinetics are cell density dependent. Rehydrated culture filtrate was combined with various concentrations of *P. fluorescens* and the first order degradation rate (k") was measured for each concentration. As shown in Figure 4.5, the CCl_4 transformation rate, as measured by the k" value, increased until the cell density was 7.5×10^7 CFU per mL. Further increases in cell concentration did not result in an increase in transformation rate. Thus, CCl_4 transformation exhibited saturation kinetics with respect to cell concentration.

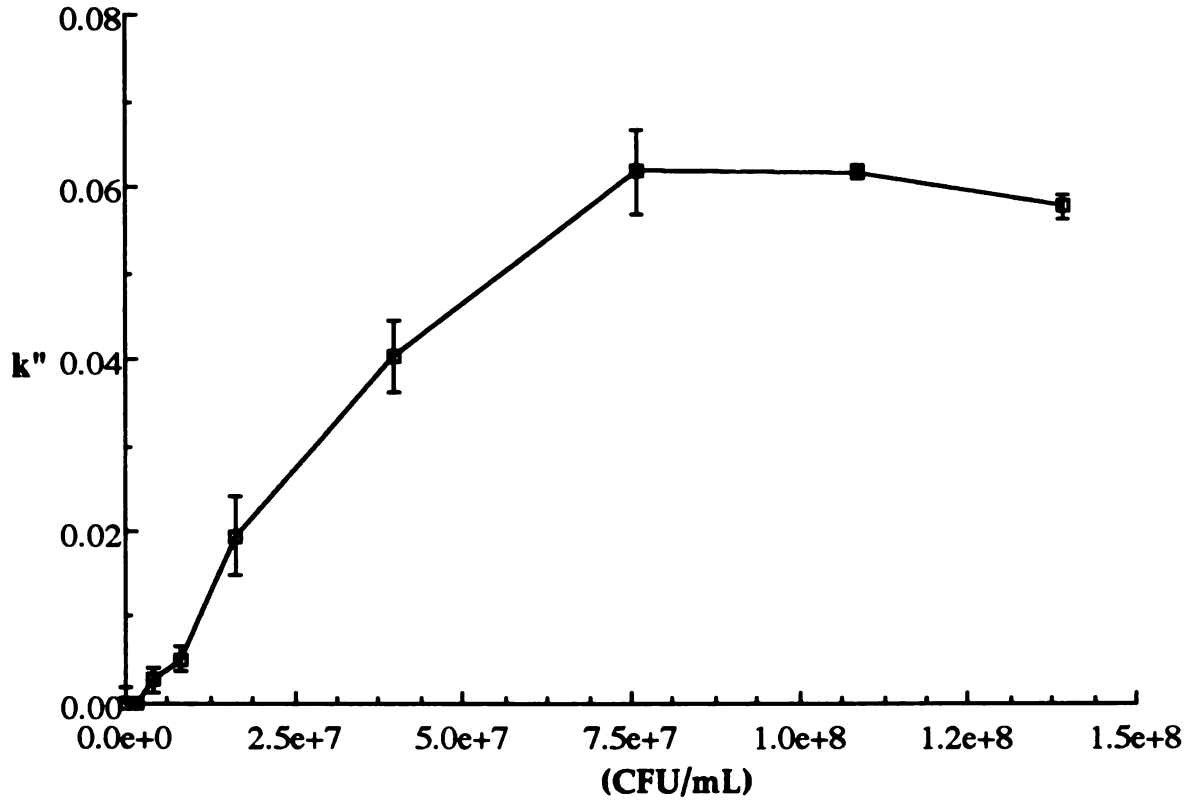

Figure 4.5. The effect of cell density on transformation rates. Secreted factor was added as freeze dried 10,000 MW filtrate from strain KC grown in SGW medium to various concentrations of *P. fluorescens* cells. Error bars represent the standard deviation of triplicate samples.

pH optimum for CCl_4 transformation. The *P. fluorescens* bioassay was used to establish the pH range for CCl_4 transformation by the secreted factor. As shown in figure 4.6, the range of pH values over which CCl_4 transformation can occur is broad. The pH optimum for CCl_4 transformation is approximately 8.5 as measured by the first order rate coefficient for CCl_4 transformation. Rates of CCl_4 transformation were lowest near pH 6.5 and 10.

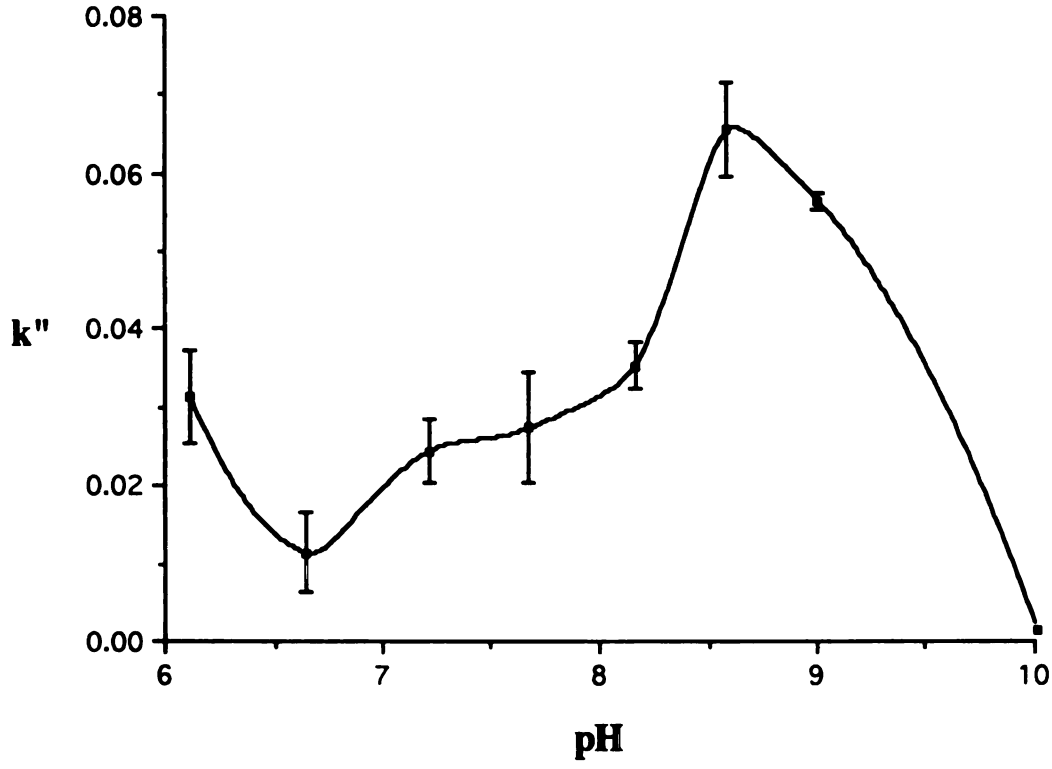

Figure 4.6. pH optimum of CCl_4 transformation. Rates of CCl_4 transformation are presented as the first-order-rate coefficient in the *P. fluorescens* bioassay. Error bars represent the standard deviation of triplicate samples.

Regeneration of activity by organisms indigenous to aquifer material. As shown in Table 4.4., significant CCl_4-removal was obtained when 10,000 MW filtrate was mixed with a slurry of biostimulated Hanford aquifer material (test sample 6). Approximately 32% of the initial CCl_4 mass (0.5 μg) was removed in two days. Addition of the supernatant factor without biostimulation (acetate and nitrate addition) failed to promote CCl_4-removal beyond that of the sterile control. Biostimulation of the indigenous flora without addition of supernatant factor also failed to bring about CCl_4-removal beyond that of the sterile control. No chloroform was detected in any of the samples with a method detection limit of 2 μg/L..

Table 4.4.. Transformation of CCl_4 in Hanford aquifer solid slurries.

Test sample	CCl_4 removed after 48 hr. incubation (μg) [a]
1) Sterile Control	0.05 ± 0.01 [b]
2) Sterile Control + KC Supernatant Factor	0.05 ± 0.05
3) Indigenous Flora	0.04 ± 0.03
4) Indigenous Flora + KC Supernatant Factor [c]	0.06 ± 0.02
5) Stimulated Indigenous Flora [d]	0.01 ± 0.05
6) Stimulated Indigenous Flora + KC Supernatant Factor	0.16 ± 0.01

[a] An initial CCl_4 mass of 0.5 μg was added to all samples. Final CCl_4 mass was determined by heating to 70 °C to release solid bound CCl_4.
[b] ± values represent the standard deviation of three independent samples
[c] KC supernatant factor was added as 2 mL of 10,000 MW filtrate from an actively transforming culture of *P. stutzeri* KC grown in SGW medium at pH 8.3
[d] Supplemented with two pulse additions of 800 mg/L acetate and 800 mg/L nitrate.

Transport of the secreted factor through aquifer material. Transport of secreted factor through aquifer material was evaluated by pumping 10,000 MW filtrate from SGW-grown strain KC through columns packed with aquifer solids from Hanford, WA. and Schoolcraft, MI. Figure 4.7a illustrates the breakthrough profile of ^3H_2O and secreted factor for Schoolcraft aquifer material as quantified by the CPM/CPM° ratio and the ratio of influent to effluent first-order bioassay rates using *P. fluorescens* . Figure 4.7b demonstrates the breakthrough profile of ^3H_2O and the secreted factor through Hanford aquifer material. The breakthrough profile for the tritiated water was similar to

that of secreted factor in both of the aquifer solid systems tested, indicating that the secreted factor was not retarded in either of these aquifer materials.

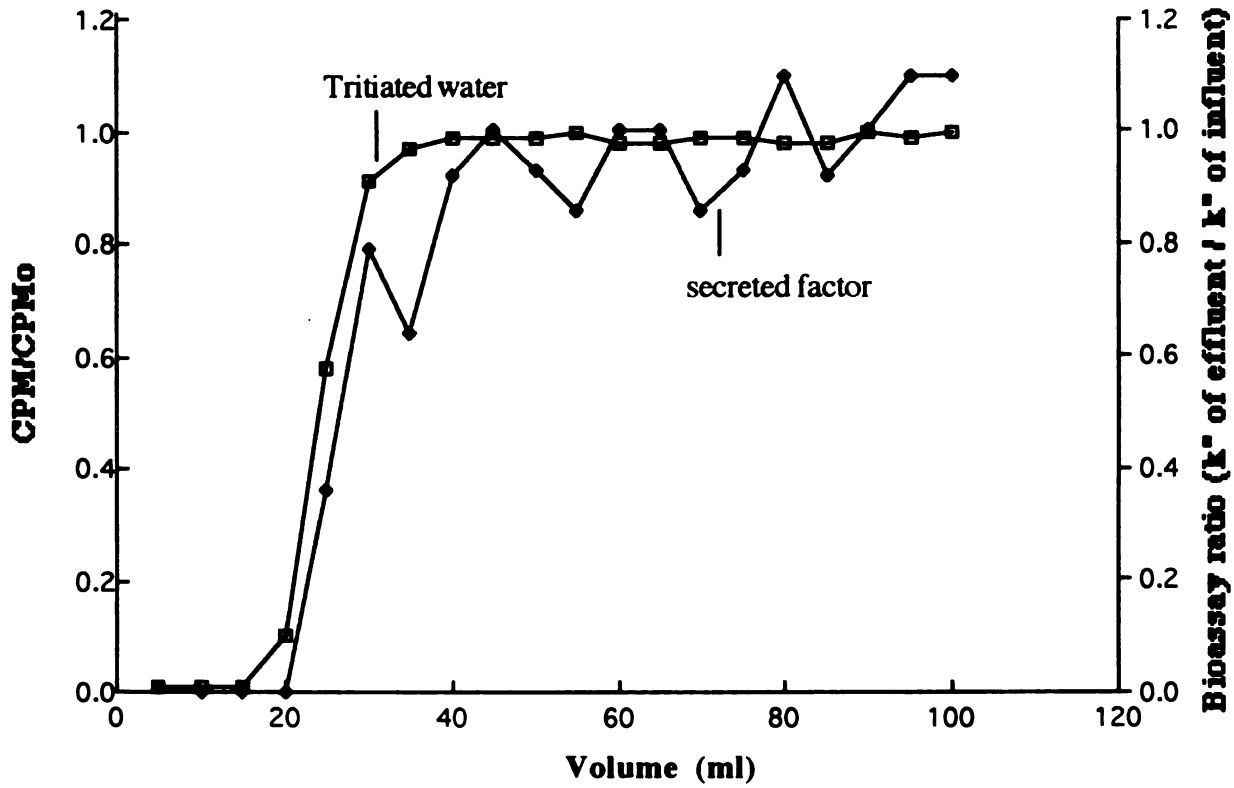

Figure 4.7a. Breakthrough profile for tritiated water and the secreted factor in a column packed with Schoolcraft, MI, aquifer material. Water was pumped through the column at a flow rate of 2.5 mL/min.

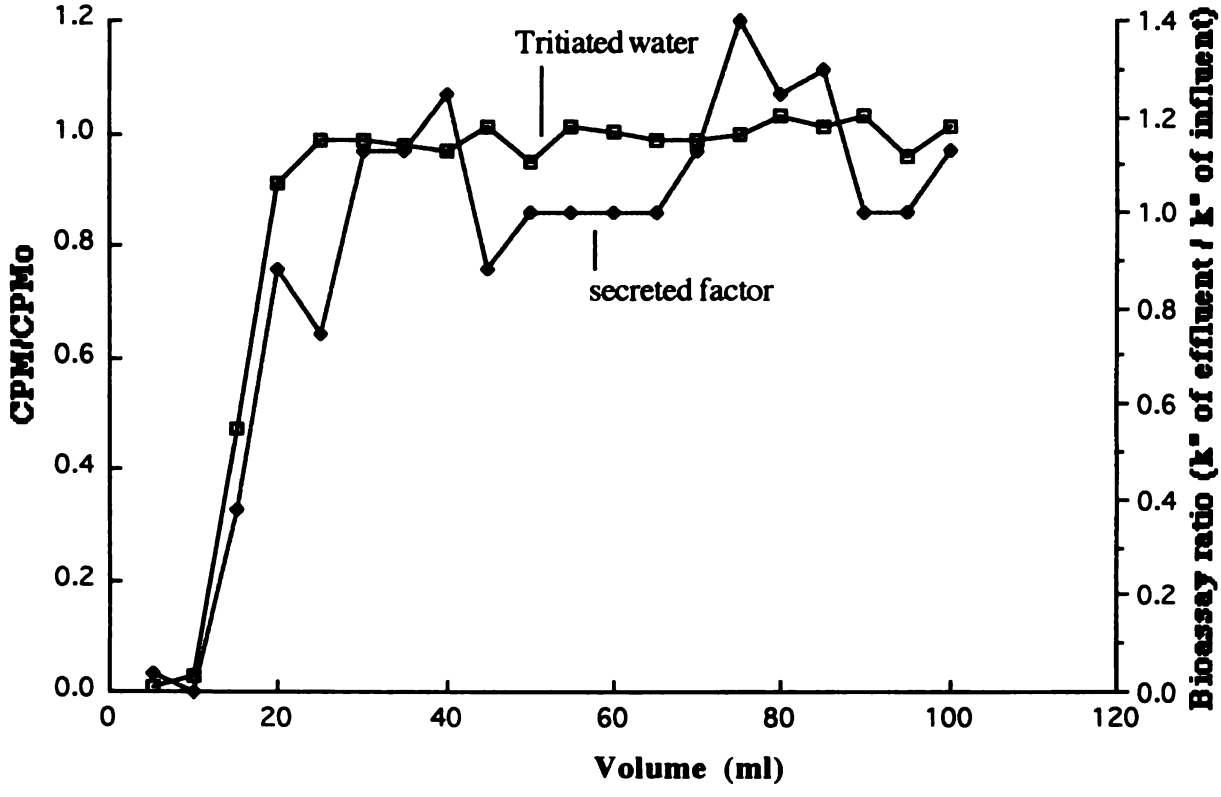

Figure 4.7b. Breakthrough profile for tritiated water and the secreted factor in a column packed with Hanford, WA, aquifer material. Water was pumped through the column at a flow rate of 2.5 mL/min.

DISCUSSION

My results indicate that rapid CCl_4 transformation is obtained when diverse microbial cell types are combined with the secreted factor produced by *P. stutzeri* KC. Previous research has established that the transformation of CCl_4 by strain KC is linked to the trace metal scavenging activities of the cell, and the role of iron was especially important [5,10,15]. In fact, the hypothesis that iron-scavenging agents, such as siderophores, might be involved in the transformation led me to speculate that organisms other than strain KC might be able to transform CCl_4 in the presence of the secreted factor. Cross

reactivity of siderophores between different bacterial species is well known [3, 9, 11]. The finding that mixtures did result in CCl_4 transformation indicates that cross reactivity does occur. However, the role of trace metal scavenging systems is unclear - although organisms grown in iron-rich medium exhibited no evidence of extracellular iron-scavenging activity, as measured with a universal chemical assay for siderophores [12], they were still able to transform CCl_4 in the presence of the secreted factor produced by strain KC. Nevertheless, the patterns of growth of *P. stutzeri* KC and production of the secreted factor [Figure 4.2], suggest that the factor does participate in trace-nutrient delivery. A very rapid initial phase of growth was observed during which no secreted factor production occurred. However, at approximately 12-hours of growth, the rate of protein increase slowed and secreted factor production accelerated. This pattern suggests that the factor is produced due to the limitation of a required nutrient or metal. To further test this concept, iron concentrations and siderophore activity should be quantified throughout the growth cycle. Understanding of iron uptake patterns could shed some light on questions regarding the natural role of this factor.

The transformation of CCl_4 by *P. stutzeri* KC appears somewhat analogous to reduction of nitroaromatic compounds mediated by *Streptomyces* sp. strain Tu 2484 exudates. Secondary metabolites cinnaquinone and dicinnaquinone, secreted during the growth phase of strain Tu 2484, have been shown to mediate electron transfer between hydrogen sulfide and various nitrobenzenes [8]. The secreted factor produced by strain KC may play a similar role, possibly functioning as a mediator of electron transfer between the cell and the CCl_4 molecule. Evidence supportive of such a hypothesis includes the following: (i) formate is one of the nonvolatile products of CCl_4 transformation by strain KC [6], and this product can only be generated by a two-electron reduction of CCl_4; (ii) actively metabolizing cells are required - no CCl_4 transformation is observed for late stationary phase cells [6,16], cells that have not been stimulated by growth substrate

addition [Table 4.2], and autoclaved cells with or without secreted factor(s) (Figure 4.4); (iii) certain oxidizing agents such as hydrogen peroxide [15] quench the reaction (although oxygen appears only to inhibit it - Figure 4.3); and (iv) rates of transformation by the secreted factor alone are significantly slower than in the presence of cells (Table 4.1). This hypothesis needs further support as proof of electron flux between the secreted factor and CCl_4 has not been demonstrated. Additionally, it would be interesting to determine if combination of the secreted factor with an abiotic electron donor such as titanium citrate would result in CCl_4 transformation. This type of experiment could help solidify the hypothesis that electron flux is involved and also provide evidence as to the role of the cell in this transformation process.

The inhibition of the CCl_4 transformation reaction by oxygen presents an interesting conflict in data between the work presented in this chapter and work reported by Lewis and Crawford. Lewis and Crawford [10] reported limited transformation of CCl_4 by cultures of strain KC grown under an oxygen headspace. However, the differences in the reported results may be attributable to differences in experimental procedures. In Figure 4.3, the time-frame of the CCl_4 transformation experiment was only a few hours, while Lewis and Crawford monitored CCl_4 transformation for 72 hours during the growth of *P. stutzeri* KC. Additionally, CCl_4 transformation was observed in their cultures after dissolved O_2 levels began to drop. The limited extent of CCl_4 transformation observed in the growing cultures may have occurred in areas of the culture medium where the metabolic rate of the growing cells was sufficient to drop localized dissolved O_2 levels to the point where CCl_4 transformation would occur. Despite the differences observed in the experimental systems, it is impossible to discount Lewis and Crawford's finding that product distribution in the aerobically grown cultures differed significantly from that in the anaerobic cultures. Under aerobic conditions, a larger proportion of the transformed CCl_4 was found converted to CO_2 than in the anaerobically grown cultures. This may

result from competition for the tri-cloromethyl radical between O_2 and other reacting species. Clearly, it would have been interesting to determine if a product distribution change existed between the biassay and whole cultures of *P. stutzeri* KC. The removal of a large portion of supernatant constituents by ultrafiltration may have resulted in a "cleaner" reaction, possibly resulting in a larger portion of the CCl_4 being converted to CO_2. This data may have helped to refute or support the hypothesis of a trichloromethyl radical intermediate.

The present work presents a generally favorable outlook for use of the secreted factor in field applications. Use of the secreted factor in combination with biostimulated indigenous populations may provide the benefits of both bioremediation and biostimulation. As with bioaugmentation, kinetic and pathway control are possible because the performance of the secreted factor can be studied and optimized in the laboratory. The pH range for CCl_4 transformation was broad (Figure 4.6), but it was optimal at a moderately alkaline pH values that are close to the values needed for iron limitation and efficient production of the factor. I detected no chloroform in any of the supernatant/cell combinations studied. In all likelihood, CCl_4 is transformed by the secreted factor faster than chloroform can be produced by a parallel pathway. As shown in Table 4.4, the amount of CCl_4 removed from slurries of Hanford aquifer material that were both biostimulated and inoculated with secreted factor was significantly more than the CCl_4 removed by biostimulation alone. Because the cells required to regenerate the secreted factor can be native to the contaminated site, many of the ecological and transport issues raised by the introduction of non-native organisms are avoided. Transport of the secreted factor is still necessary, but we observed no difficulties in moving it through aquifer material (Fig. 4.7a and 4.7b). Other data supporting possible application of the secreted factor are its ability to be concentrated by freeze drying (Table 4.3), and its favorable storage properties after freeze-drying. Its stability in water is a possible

drawback to application and will need further evaluation, as only limited stability was obtained in medium D and SGW medium (Table 4.2).

In the following chapter, these studies are further expanded upon by the identification of a cell type that is incapable of regenerating the secreted factor. Additionally, evidence is provided which supports the finding that the cell may function to transfer electrons to the CCl_4 molecule in a reaction mediated by the secreted factor.

REFERENCES

1. **APHA/AWWA/WEF.** 1992. Standard methods for the examination of water and wastewater. American Public Health Assoc. Washington, DC.

2. **Armenante, P.M., N. Pal, and G. Lewandowski.** 1994. Role of mycelium and extracellular protein in the biodegradation of 2,4,6-trichlorophenol by *Phanerochaete chrysosporium. Appl. Environ. Microbiol.* **60:** 1711-1718.

3. **Buyer, J.S. and J. Leong.** 1986. Iron transport-mediated antagonism between plant growth promoting and plant-deleterious *Pseudomonas* strains. *J. Biol. Chem.* **261:** 791-794.

4. **Criddle, C.S., J.T. DeWitt, and P. L. McCarty.** 1990. Reductive dehalogenation of carbon tetrachloride by *Escherichia coli* K-12. *Appl. Environ. Microbiol.* **56 :** 3247-3254.

5. **Criddle, C.S., J.T. DeWitt, D. Grbić-Galić and P. L. McCarty.** 1990. Transformation of carbon tetrachloride by *Pseudomonas* sp. strain KC under denitrification conditions. *Appl. Environ. Microbiol.* **56:** 3240-3246.

6. **Dybas, M.J., G. M. Tatara, and C.S. Criddle.** 1994. Localization and characterization of the carbon tetrachloride transforming activity of *Pseudomonas* sp. strain KC. *Appl. Environ. Microbiol.* **61:** 758-762.

7. **Egli, C., T. Tschan, R. Scholtz, A.M. Cook, and T. Leisinger.** 1988. Transformation of tetrachloromethane to dichloromethane and carbon dioxide by *Acetobacterium woodii.* Appl. Environ. Microbiol. **54:** 2819-2823.

8. **Glaus, M.A., C.G. Heijman, R.P. Schwarzenbach, and J. Zeyer.** 1992. Reduction of nitroaromatic compounds mediated by *Streptomyces* sp. exudates. *Appl. Environ. Microbiol.* **58:** 1945:1951.

9. **Hohnadel D. and J..M. Meyer.** 1988. Specificity of pyoverdine-mediated iron uptake among fluorescent *Pseudomonas* strains. *J. Bacteriol.* **170:** 4865-4873.

10. **Lewis, T.A. and R.L. Crawford.** 1993. Physiological factors affecting carbon tetrachloride dehalogenation by the denitrifying bacterium *Pseudomonas* sp. strain KC. *Appl. Environ. Microbiol.* **59:** 1635-1641.

11. **Morris, J., D.J. O'Sullivan, M. Koster, J. Leong, P. J. Weisbeek, and F. O'Gara.** 1992. Characterization of fluorescent siderophore-mediated iron uptake in *Pseudomonas* sp. strain M114: evidence for the existence of an additional ferric siderophore receptor. *Appl. Environ. Microbiol.* **58:** 630-635.

12. **Schwyn, B. andJ.B. Neilads.** 1987. Universal chemical assay for the detection and determination of siderophores. *Anal. Biochem.* **160:** 47-56.

13. **Semprini, L., G. D. Hopkins, P.L. McCarty, and P.V. Roberts.** 1992. In-situ transformation of carbon tetrachloride and other halogenated compounds resulting from biostimulation under anoxic conditions. *Environ. Sci. Technol.* **26:** 2454-2461.

14. **Sittig, M. (ed.).** 1985. Handbook of toxic and hazardous chemicals and carcinogens, 2nd. Noyes Publications, New York.

15. **Tatara, G.M., M.J. Dybas, and C.S. Criddle.** 1993. Effects of medium and trace metals on the kinetics of carbon tetrachloride transformation by *Pseudomonas* sp. strain KC. *Appl. Environ. Microbiol.* **59:** 2126-2131.

CHAPTER 5

ROLE OF CELL MEMBRANES IN THE TRANSFORMATION OF CARBON TETRACHLORIDE BY *PSEUDOMONAS STUTZERI* KC

ABSTRACT

Previous research has established that the transformation of CCl_4 by *Pseudomonas stutzeri* KC proceeds via a complex mechanism involving both a secreted (cell-free) factor and cell-associated factors. The combination of the secreted factor and a diverse range of cell types results in rapid CCl_4 transformation. The present study provides further insight into the cell associated factor(s) required for CCl_4 transformation. The combination of the secreted factor with fermenting cells of *Escherichia coli* resulted in rapid CCl_4 transformation, but a strictly fermenting bacterium, *Lactobacillus acidophilus* , did not mediate rapid CCl_4 transformation. *L. acidophilus* lacks a membrane bound electron transport chain and cytochromes, suggesting participation of these constituents in regeneration of the secreted activity. Crude cell membranes supplemented with secreted factor and NADH rapidly transformed CCl_4, demonstrating the need for a membrane-associated redox component(s). The metalo-center inhibitors cyanide and pyridine both inhibited CCl_4 transformation. Chloramphenicol also inhibited CCl_4 transformation. These data led to the development a new model which posits that CCl_4 transformation activity requires both a secreted factor and a non-respiratory membrane-associated electron transport agent.

INTRODUCTION

The transformation of carbon tetrachloride (CCl_4) by *Pseudomonas stutzeri* KC involves both a cell-associated and a small (< 500d) cell-free component [4]. Previous studies demonstrated that rapid CCl_4 transformation is obtained when diverse microbial cell types are combined with the secreted factor produced by *P. stutzeri* KC [9]. Formate, a two electron reduction product of CCl_4, is an end product of the transformation, suggesting that the secreted factor is a reductant capable of a two-electron transfer [4]. One possible hypothesis is that regeneration of the CCl_4 transforming factor secreted by strain KC is reduced by one or more of the respiratory chain components.

In this chapter, I describe experiments designed to assess the role of electron transport in regeneration of the secreted factor. Inhibitor studies were performed with *Pseudomonas fluorescens* , an organism capable of rapid CCl_4 transformation in the presence of the secreted factor produced by strain KC [4,9]. Additionally, a simplified electron transport system consisting of NADH and crude cell membranes of *P. stutzeri* KC is shown to reconstitute CCl_4 transformation activity. Finally, a revised model is postulated which more accurately describes CCl_4 transformation.

MATERIALS AND METHODS

Chemicals. CCl_4 (99% purity) , sodium cyanide, and sodium azide, were obtained from Aldrich Chemical Co., Milwaukee, Wis. INT (2-(p-iodo-phenyl)-3-(p-nitrophenyl)-5-phenyl tetrazolium chloride), rotenone (1,2,12,12a-tetrahydro-8,9-dimethoxy-2-(1-methylethenyl-[1]benzo-pyrano[3,4-b]furo[2,3h]-[1]-benzopyran-6(6H)-one), quinicrine dihydrochloride, dicumarol (3,3'-methylenebis[4-hydroxy-2H-1-benzypyran-2-one]), HOQNO (2-heptyl-4-hydroxyquinoline N-oxide), DCCD (N,N'-dicyclohexyl-carbodiimide), DNP (2,4-dinitrophenol), and chloramphenicol were obtained from Sigma Chemical Co. All chemicals used in media preparation were ACS reagent grade (Aldrich or Sigma Chemical Co.). All water used in reagent and inhibitor preparation was deionized 18 Mohm resistance or greater.

Media. Hanford simulated groundwater (SGW) was prepared as described in Chapter 3. Lactobacilli broth AOAC (Difco) was prepared according to the manufacturer's instructions. The resulting medium was autoclaved at 121°C for 20 minutes and transferred to an anaerobic glove box (Coy Laboratories, Ann Arbor, MI) for degassing.

Preparation of partially purified culture supernatant. Preparation of filtered and lyophilized culture supernatant was prepared as described in Chapter 4.

Preparation of crude cell membranes. Two liters of *P. stutzeri* KC (ATCC deposit no. 55595, DSM deposit no. 7136) culture was grown from a 1% inoculum in SGW medium under aerobic conditions in 4L erlenmeyer flasks. 2 L of culture was transferred to 250 mL Nalgene® centrifuge tubes and centrifuged at 8,200 x g at 4 °C. Pellets were resuspened in 50 mL of 50 mM potassium phosphate buffer (pH 8.0) and were pooled together into 2 centrifuge tubes containing 200 mL of cell resuspension each. These

resuspended pellets were centrifuged again at 8,200 x g for 15 minutes at 4 °C. The resulting cell pellets had a total mass of 5 g and were resuspended in 12 mL of 50 mM phosphate buffer (pH 8.0). The washed and resuspended cell pellet was sonicated for 10 min. at 1 sec. bursts with 50% time on and 50 % time off. DNAse I was added at final concentration of 250 ng/mL, and the lysate was incubated for 30 minutes at 20°C. The lysate was centrifuged at 8,200 x g, for 10 minutes at 4 °C to remove unbroken cells. The cell-pellet was discarded and the lysate centrifugation process was repeated. The resulting lysate was ultracentrifuged at 150,000 x g at 4 °C for 120 min. Following ultracentrifugation, the cytoplasmic fraction was decanted and the membrane fraction was gently resuspended in 12 mL of 50 mM potassium phosphate buffer pH 8.0. The crude cell membrane and cytoplasmic fractions were used immediately in a CCl_4 transformation assay with partially purified secreted factor.

CCl_4 transformation assays with crude cell membrane preparations. Lyophilized 500 MW filtrate was rehydrated to its original volume (5 mL) as follows. 4.5 mL of 500 MW filtrate was transferred to 28 mL aluminum seal tubes (Bellco Glass), then supplemented with 500 μL of fresh cytoplasmic fraction, 500 μL of fresh membrane preparation, or 500 μL of 50 mM potassium phosphate buffer. Test samples were made anoxic by passage through the interlock of an anaerobic glove box. ß-NADH (Sigma) was added to select samples to give a final concentration of 20 μM. The serum tubes were sealed under anoxic conditions with Teflon-lined butyl rubber septa (West) and aluminum crimp seals (West). CCl_4 transformation was monitored as described in Chapter 2.

Measurement of electron transport system activity. Electron transport activity measurements were performed by the method of Zimmerman et al. [10] and Trevors et al. [14]. A 10 mL quantity of sample was poured into sterilized serum tubes, and 1 mL of a

0.2% (w/v) tetrazolium dye was added to each tube. At various time intervals, 1 ml aliquots were removed from the sample tubes. The 1 mL aliquots were extracted with 10 mL of HPLC grade methanol, filtered through 0.22 μm nylon filters, and measured spectrophotometrically at 480 nm. CCl_4 transformation was monitored as described previously.

CCl_4 transformation with *Lactobacillus acidophilus*. *L. acidophilus* (ATCC no. 4356) was obtained from the American Type Culture Collection. *L. acidophilus* was grown anaerobically in Lactobacilli broth AOAC in 120 mL serum bottles at 35 °C. Cultures of *L. acidophilus* were transferred to an anaerobic glove box and dispensed into 40 mL centrifuge tubes. Cells were collected by centrifuging at 12,100 x g for 5 minutes, wasting the culture supernatant, and resuspending the pellet in 4 mL of anoxic Lactobacilli broth AOAC. A 0.5 mL sample of cell suspension (concentrated ten fold by centrifugation) was added to 4.5 mL of filtered supernatant and assayed for CCl_4 transformation in 28 mL aluminum seal tubes as previously described.

Lactate production was measured using the lactate reagent kit from Sigma Chemical Co. A standard curve was prepared using lactate over the concentration range 0-10 mg/mL, and measuring the absorbance at 540 nm.

CCl_4 transformation with fermenting *E. coli*. *E. coli* K-12 (ATCC no. 10798) was obtained from the culture collection of the Microbiology Department at Michigan State University. *E. coli* was grown at 35°C under strictly fermenting conditions with 3 g/L of glucose as the carbon and energy source in SGW medium. SGW medium for the growth of *E. coli* under fermenting conditions was prepared nitrate free to avoid the production of anaerobic respiratory chains. Cultures were grown to an OD_{660} of approximately 0.18, transferred to an anaerobic glove box, dispensed into 40-mL Nalgene centrifuge tubes,

fitted with butyl rubber septa, and centrifuged at 12,100 x g for 5 min. The culture supernatant was decanted, and the pellet was resuspended in 4 mL of nitrate free anoxic SGW medium with glucose as the electron donor. A 0.5 mL sample of cell suspension (concentrated ten fold by centrifugation) was added to 4.5 mL of filtered supernatant and assayed for CCl_4 transformation as described previously.

Inhibitor Studies. Relevant information pertaining to the concentration and proper solvent for each inhibitor is summarized in Table 1. All inhibitor stock solutions were prepared fresh daily at concentrations sufficiently high so that a minimal volume of solvent was added in the inhibitor studies. This was to minimize the chances of secondary effects attributable to solvent addition. Appropriate controls were also prepared to ensure that solvents themselves were not responsible for inhibition.

Cultures of *P. fluorescens* were grown aerobically in SGW medium to a desired optical density of $OD_{660} = 0.15$, a value chosen to ensure that the cultures were in the exponential stage of growth. Cultures were dispensed into 40-mL Nalgene® centrifuge tubes. Cells were collected by centrifuging at 12,100 x g for 5 minutes , wasting the culture supernatant, and resuspending the pellet in 4 mL of SGW medium at pH 8.2. A 0.5 mL sample of cell suspension (concentrated ten fold by centrifugation) was added to 4.5 mL of partially purified culture filtrate in a 28 mL serum tube and the desired inhibitor or solvent was added. The resulting test samples were made anoxic by transfer through the interlock of a Coy anaerobic glove box. Tubes were sealed under anoxic conditions and CCl_4 transformation was monitored as previously described.

Modeling. The first-order-rate coefficient or k" value was calculated as described previously in Chapter 4.

Table 5.1. Summary of inhibitors used

Chemical Inhibitor	Concentration Range (μM)[a]	Solvent	Inhibition Site or Action
Rotenone	10 - 1,000	Acetone	NADH Dehydrogenase
Quinacrine dihydrochloride	1 - 1,000	Water	Flavins
Dicumarol	1 - 50	Pyridine	Quinones
HOQNO	0.7 - 50	Ethanol	Cytochrome *b*
NaCN (cyanide)	10 - 3,000	Water	Cytochrome *c* oxidase
NaN_3 (azide)	20 - 20,000	Water	Cytochrome *c* oxidase
DCCD	1 - 100	Acetone	ATPase (Fo subunit)
DNP	1 - 500	Acetone	Uncoupler
Chloramphenicol	100 - 200	Ethanol	Bacteriostatic Agent

a. The concentrations of inhibitors used in this study were chosen based on concentrations referenced in a similar study performed by Arnold et al [2].

RESULTS

Transformation of CCl_4 by crude cell membrane preparations. Crude cell membrane and cytoplasmic fractions were prepared to determine if membrane bound or cytoplasmic proteins provide reducing equivalents to the secreted factor produced by *P. stutzeri* KC. NADH was added to determine if it enhances transformation or if it can provide reducing equivalents to the secreted factor with an additional mediator protein. Freshly prepared membrane and cytoplasmic fractions were combined with the secreted factor with and without NADH. As shown in Figure 5.1, rapid CCl_4 transformation occurred when the secreted factor was combined with crude membrane preparation from *P. stutzeri* KC in

the presence of 20 μM NADH. Transformation of CCl_4 also occurred when the secreted factor was combined with crude membranes without NADH present. However, the rates were not as fast as with NADH. No CCl_4 transformation was observed when the secreted factor was combined with the cytoplasm of *P. stutzeri* KC cells with or without NADH present. The secreted factor did not transform CCl_4 when combined with NADH, thus suggesting that a membrane-bound component mediates transfer of reducing equivalents from NADH to the secreted factor.

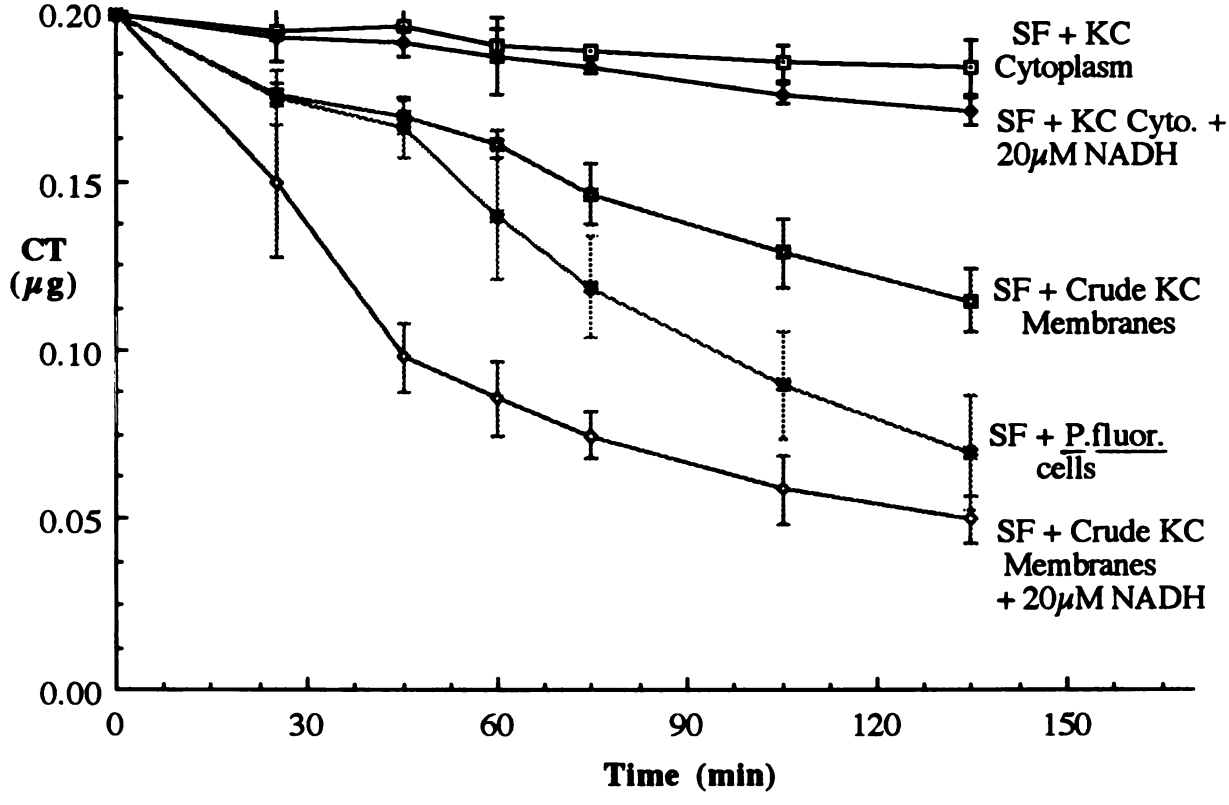

Figure 5.1. Transformation of CCl_4 with crude cell membrane preparations from *P. stutzeri* KC. Error bars represent the standard deviation of triplicate samples.

INT-Formazan inhibits CCl_4 Transformation. An active electron transport system is an almost universal component of respiring organisms, which reduces INT (2-(p-iodo-phenyl)-3-(p-nitrophenyl)-5-phenyl tetrozolium chloride) to INT-formazan [12]. The

conversion of INT to INT-formazan was measured as a means of determining the effectiveness of inhibition of electron transport system activity. As shown in Figure 5.2, the production of INT-formazan inhibits CCl_4 transformation by cultures of *P. stutzeri* KC. As a result, INT-formazan production could not be utilized in the inhibitor studies a means of determining the effectiveness of a given inhibitor.

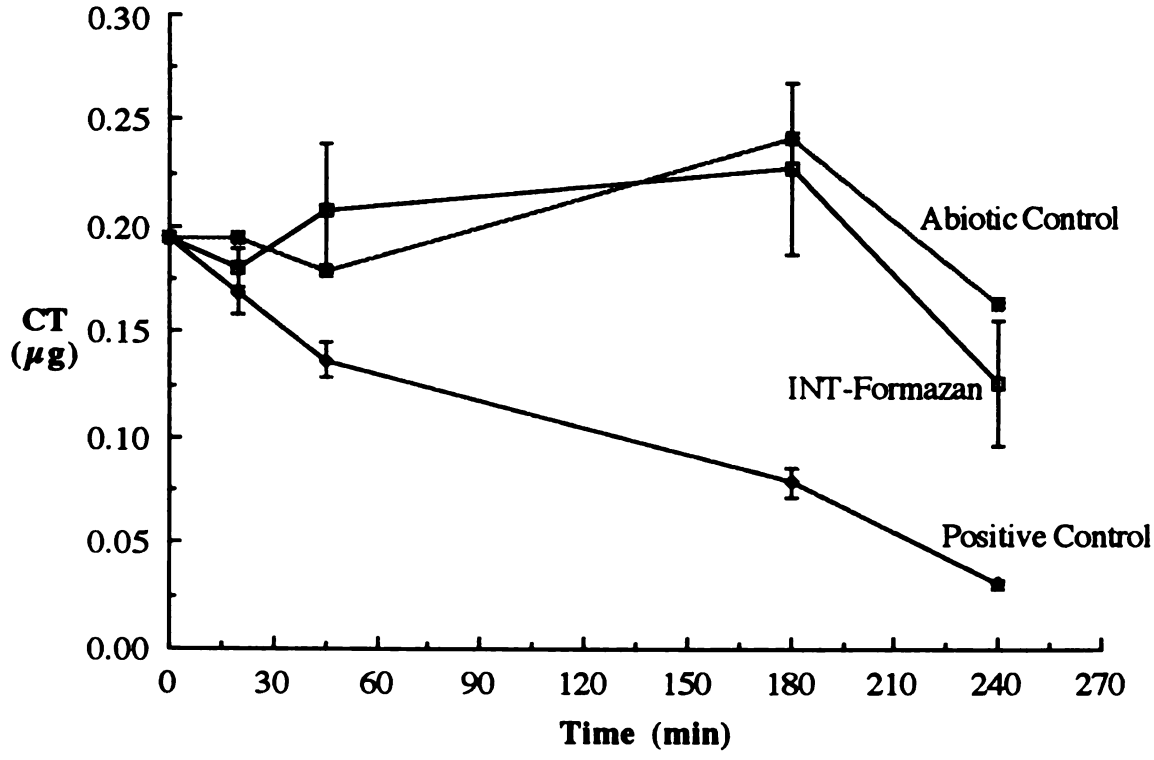

Figure 5.2. Effect of INT-Formazan on CCl_4 transformation by *P. stutzeri* KC. Error bars represent the standard deviation of triplicate samples.

Comparison of CCl_4 transformation with *B. subtilus* and *L. acidophilus*. *L. acidophilus* was combined with the secreted factor from *P. stutzeri* KC to determine if a strictly fermenting bacterium could regenerate the secreted factor to result in CCl_4 transformation. As shown in Figure 5.3, *L. acidophilus* does not rapidly transform CCl_4 in the presence of the secreted factor. In fact, rates for the secreted factor alone were very similar to those observed for the secreted factor in combination with *L. acidophilus*.

Since *L. acidophilus* is a gram positive organism, *B. subtilus* was used a positive control. Cultures of *L. acidophilus* were actively fermenting glucose to lactate (data not shown) during the transformation assay. Therefore, the alkaline pH necessary for CCl_4 transformation was not inhibitory to the metabolism of *L. acidophilus*.

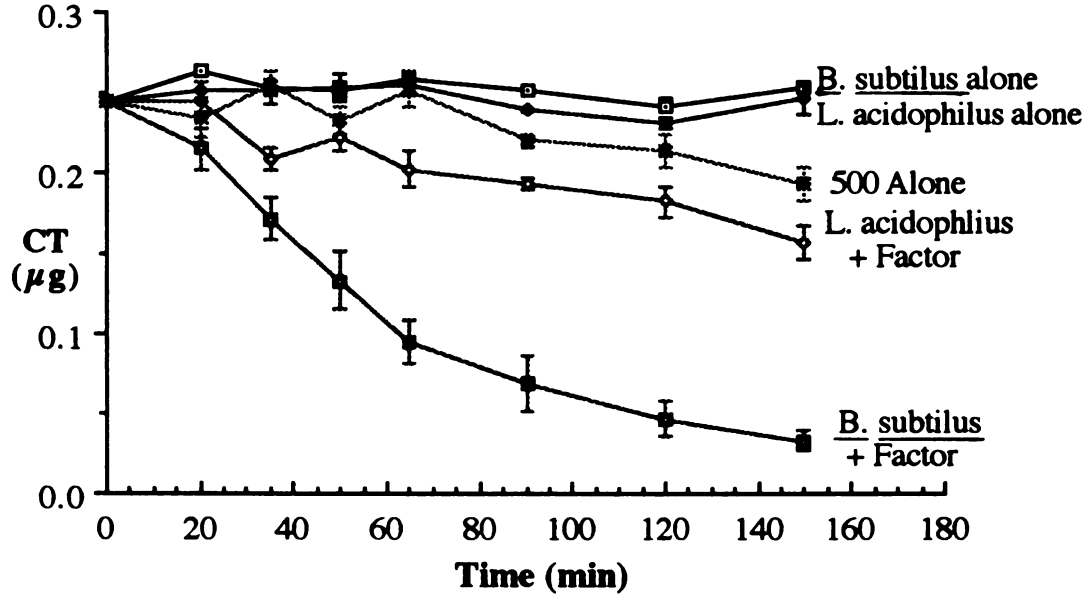

Figure 5.3. Comparison of CCl_4 transformation with *B. subtilus* and *L. acidophilus* when combined with the secreted factor from *P. stutzeri* KC. Error bars represent the standard deviation of triplicate samples.

Combination of the secreted factor with fermenting *E. coli*. *E. coli* was grown under strictly fermenting conditions to determine if recharge of the secreted factor required respiring cells (either aerobically or anaerobically). The combination of *E. coli* under fermenting conditions with the secreted factor from strain KC resulted in CCl_4 transformation (Figure 5.4).

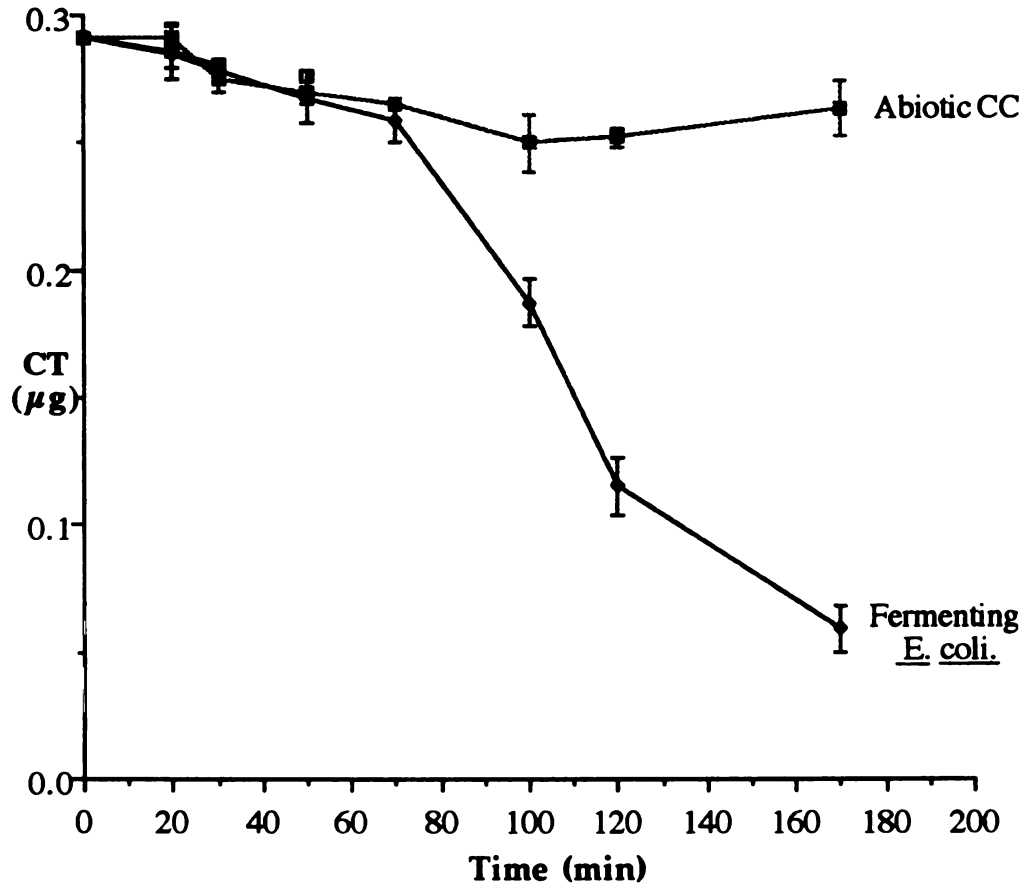

Figure 5.4. Transformation of CCl_4 by strictly fermenting *E. coli* combined with the secreted factor from *P. stutzeri* KC. Error bars represent the standard deviation of triplicate samples.

Table 5.2. Effect of electron transport inhibitors as measured by first-order rate coefficients.

Chemical Inhibitor	Concentration Range (μM)[a]	k" (min^{-1})[a]	Inhibition
Rotenone	0 - 1,000	(0.08 ± 0.01) - (0.06± 0.01)	Slight
Quinacrine dihydrochloride	0 - 1,000	(0.06± 0.01) - (0.05± 0.1)	No
Dicumarol	0 - 50	(0.08±0.01) - (0.07± 0.01)	No
HOQNO	0 - 50	(0.07±0.02) - (0.06±0.01)	No
NaCN (cyanide)	0 - 3,000	(0.07±0.01) - (0.00)	Yes
NaN_3 (azide)	0 - 20,000	(0.07±0.01) - (0.05±0.01)	Slight
DCCD	0 - 100	(0.06±0.01) - (0.08±0.01,	No
DNP	0 - 500	(0.06±0.01) - (0.09±0.01)	No
Chloramphenicol	0- 200	(0.19±0.02) - (0.05±0.01)[b]	Yes
Pyridine	0 - 25 μL	(0.08 ± 0.01) - (0.02±0.01,	Yes

[a] first-order-rate coefficient (k") values represent the average of triplicate samples, standard deviations are not shown.

[b] rate calculated on a whole culture of *P. stutzeri* KC

Inhibitors of electron transport. Specific respiratory chain inhibitors were evaluated in order to determine if components of the respiratory electron transport system are involved in transformation of CCl_4 with the secreted factor produced by *P. stutzeri* KC. Table 5.2 shows that 1 mM rotenone slightly inhibits the CCl_4 transformation reaction. A concentration of 5 mM rotenone (data not shown) had a greater inhibitory effect on CCl_4 transformation, but the rotenone was added in 500 μL of acetone, which also significantly inhibited CCl_4 transformation. Lower concentrations of rotenone did not inhibit the CCl_4 transformation reaction. Quinicrine dihydrochloride, an inhibitor of flavins, does

not inhibit the CCl_4 transformation reaction of *P. fluorescens* combined with the secreted factor from *P. stutzeri* KC. Dicumarol also does not affect the CCl_4 transformation reaction. However, pyridine which was the solvent used for the addition of dicumarol significantly inhibited CCl_4 transformation. HOQNO, a cytochrome *b* inhibitor, does not inhibit CCl_4 transformation, and either does DCCD, an inhibitor of the Fo subunit of ATPase. DNP, which is an uncoupler and renders the cytoplasmic membrane permeable to H^+, also does not inhibit transformation.

Cyanide, a cytochrome oxidase inhibitor significantly inhibited the transformation of CCl_4 as shown in Table 5.2. Concentrations of cyanide as low as 10 μM completely inhibited CCl_4 transformation. Another cytochrome oxidase inhibitor, azide, only slightly inhibited CCl_4 transformation at a concentration of 2 mM. Both cyanide and azide were added in water, so solvent effects were not a concern in this experiment. Chloramphenicol, a bacteriostatic agent which blocks protein synthesis and porins significantly inhibits CCl_4 transformation at a concentration of 170 μM.

DISCUSSION

P. stutzeri KC transforms CCl_4 by a complex mechanism involving both cell-free and cell-associated factors. [4,9]. I had previously hypothesized that mediation of the CCl_4 transformation may be linked to an interaction between the secreted factor and a cell-associated factor in the electron transport system of bacteria. Three lines of evidence support the hypothesis that a membrane-associated redox component(s) is required to mediate CCl_4 transformation activity: (1) experiments with crude cell extracts, (2) inhibition of activity by INT-formazan, and (3) lack of transformation by the *L. acidophillus* system. Crude cell extract experiments established that CCl_4 transformation required a source of reducing equivalents (NADH), a cell membrane, and the secreted

factor (Figure 5.1). In addition, several previous hypotheses were refuted by this experiment. A cytoplasmic component is not required for transformation, and NADH does not reduce the secreted factor directly . Clearly, a membrane bound component is required for transfer of reducing equivalents from NADH to the secreted factor. Inhibition of CCl_4 transformation by INT-formazan suggests that an electron transport chain is required for regeneration of the secreted factor (Figure 5.2). Dehydrogenases reduce INT to INT-formazan, which is deposited as optically dense purple crystals inside the bacteria. INT-formazan may compete with the secreted factor for reducing equivalents entering an electron transport chain. *L. acidophilus*, an organism lacking any membrane bound electron transport system [5], facilitated only a very limited level of CCl_4 transformation when combined with the secreted factor (Figure 5.3).

The nature of the membrane-associated redox component was further explored in experiments with fermenting *E. coli* , which does not utilize respiratory electron transport chains for growth. Fermenting *E. coli* was capable of regenerating activity (Figure 5.4). This suggests that the membrane-associated redox component(s) involved in CCl_4 transformation may not be a respiratory chain. Not all of the electron transport chains present in *E. coli* are required respiratory electron transport-dependent ATP synthesis [6].

Additional support for the hypothesis that a non-respiratory redox component(s) is involved in the reaction was obtained from inhibitor studies. Reducing equivalents enter the respiratory chain via one of several dehydrogenases (often flavin containing enzymes, e.g. NADH and succinate dehydrogenases) and are passed sequentially down a chain of carriers which includes iron-sulfur proteins, coenzyme Q, and a series of cytochromes [2]. Table 5.2 shows that rotenone, an inhibitor of NADH dehydrogenase, only slightly inhibited CCl_4 transformation at a concentration of 1 mM. Quinicrine dihydrochloride,

an inhibitor of the flavin groups in dehydrogenases, did not inhibit CCl_4 transformation. These results argue against the idea that the secreted factor accepts reducing equivalents from a respiratory chain enzyme. If an enzyme in the respiratory chain were involved in CCl_4 transformation, then these dehydrogenase inhibitors should impact CCl_4 transformation. However, dicumarol (a quinone inhibitor) and HOQNO (a cytochrome *b* inhibitor) did not inhibit CCl_4 transformation . Therefore, a model in which the secreted factor accepts reducing equivalents from a component of the respiratory chain does not adequately describe the mechanism of CCl_4 transformation.

It could be argued that inhibition by cyanide implicates a respiratory chain cytochrome as the step where secreted factor interacts to result in CCl_4 transformation. Both bacteria and eukaryotic organisms contain cytochrome oxidase [13], and both organism types regenerated activity. Cyanide is known to inhibit cytochrome oxidase, and it strongly inhibited CCl_4 transformation. However, if cytochrome oxidase were capable of regenerating activity, it should have been blocked by inhibitors acting higher up on the electron transport chain. In addition, azide, which is a known cytochrome oxidase inhibitor, only slightly inhibited CCl_4 transformation at a concentration of 20 mM.

Pyridine also inhibits CCl_4 transformation when added in sufficient quantity. Both pyridine and cyanide bind tightly to the metal centers of many enzymes. Cyanide is a nucleophile and can attack electrophilic sites. In addition, cyanide complexes with the Fe^{2+} form of iron bound to the heme cofactor [12]. Therefore, even though cyanide is listed as an inhibitor of cytochrome oxidase, it is not a specific inhibitor of this enzyme.

DCCD, a specific inhibitor of the F_o subunit of ATPase did not inhibit CCl_4 transformation, indicating that in the short term, ATP is not involved in the transformation reaction. Additionally, a proton gradient is not required for CCl_4

transformation as DNP, an uncoupler which renders the cytoplasmic membrane permeable to H+ ions, did not inhibit CCl_4 transformation. This is not inconsistent with a previous observation by Dybas et al. [4], who observed that CCCP, which uncouples respiration from ATP synthesis, stimulated resting cells to transform CCl_4. The presence of either DNP or CCCP collapses the proton gradient and stimulates rapid electron transfer. Therefore, over the short-term, maintenance of a proton gradient and production of ATP are not necessary for regeneration of the secreted factor.

A few other clues for the mechanism of transformation were obtained. Chloramphenicol inhibited CCl_4 transformation. One possible mechanism of chloramphenicol inhibition is related to its capacity to stop translation at the ribosome level. This mechanism can be discounted because: (1) other experiments conducted with chloramphenicol indicated inhibition in a time frame too short for new protein synthesis (<10 minutes) and (2) chloramphenicol inhibited CCl_4 transformation in a bioassay with *P. fluorescens*, an organism that does not produce the secreted factor. A second inhibitory mechanism is blockage of bacterial outer membrane porin channels [3]. For the secreted factor to obtain reducing equivalents at the inner membrane and transform CCl_4 outside the bacterial cell, it must be able to freely diffuse in and out of the outer membrane porin channels which typically allow free diffusion of molecules smaller than 600MW. The small size of the secreted factor (< 500 daltons) would make this diffusion possible, unless the porin channels were blocked by chloramphenicol.

Tatara et al. [10] showed that copper is required for CCl_4 transformation by *P. stutzeri* KC. Both cyanide[1] and pyridine [8] are strong ligands to the metal centers of enzymes. Thus, one possible mode of inhibition might involve blockage of a copper containing enzyme needed for regeneration of the secreted factor. It is also possible that cells grown

in the absence or at very low copper concentrations could lack copper containing enzymes needed to regenerate or produce the secreted factor.

As a result of the data presented above, a new model is proposed for recharge of the secreted factor. The new model posits that a non-respiratory membrane-associated electron transport chain is required for regeneration of CCl_4 transformation activity. Additional research is needed to understand the role of trace metals in the regeneration of CCl_4 transformation activity. Additional work is also needed to identify the specific factor(s) that transfers reducing equivalents to the secreted factor.

REFERENCES

1. **Alexander K. and S.I. Baskin.** 1987. The inhibiton of cytochrome oxidase by diaminomaleonitrile. *Biochemica et Biophysica Acta*. **912:** 41 -47.

2. **Arnold, R. G., T.J. DiChristina, and M. R. Hoffman.** 1986. Inhibitor studies of dissimilative Fe(III) reduction by Pseudomonas sp. strain 200 ("*Pseudomonas ferrireductans*"). *Appl. Environ. Microbiol.* **52**: 281-289.

3. **Chopra, I**. 1990. Penetration of antibiotics to their target sites. *Journal of Antimicrobial Chemotherapy.* **26:** 607-609.

4. **Dybas, M.J., G. M. Tatara, and C.S. Criddle.** 1995. Localization and characterization of the carbon tetrachloride transformation activity of *Pseudomonas* sp. strain KC. *Appl. Environ. Microbiol.* **61**: 758-762.

5. **Gottshalk, G**. 1986. Bacterial Metabolism, Second Edition. Springer-Verlag, New York.

6.**Haddock, B.A. and C.W. Jones**. 1977. Bacterial respiration. *Bacteriological Reviews.* **41:** 47 - 99

7. **Shienke, A.K. W.A, Kaplan, C.L. Hamilton, J. A. Shelnutt, and R.A. Scott.** 1989. Structural and spectroscopic characterization of exogenous ligand binding to isolated factor F430 and its configuration isomers. *J. Biol. Chem.* **246:** 7267-7284.

8. **Peterson, J. N., R.S. Skeen, K. M. Amos, and B.S. Hooker.** 1994. Biological destruction of CCl4: I. experimetnal design and data. *Biotech. Bioeng*. **43**: 521-528.

9. **Tatara, G.M., M.J. Dybas, and C.S. Criddle.** 1995. Biofactor-mediated transformation of carbon tetrachloride by diverse cell types, p. 69-77. *In* R. Hinchee, A. Leeson, and L. Semprini (ed.) The Bioremediaton Series, **3(3)**, Bioremediation of Chlorinated Solvents. Battelle Press, Richland, WA.

10. **Tatara, G.M., M.J. Dybas, and C.S. Criddle**. 1993. Effects of medium and trace metals on kinetics of carbon tetrachloride transformation by *Pseudomonas* sp. strain KC. *Appl. Environ. Microbiol.* **59**:2126-2131.

11. **Trevors, J.T., C.I. Mayfield, and W.E. Inniss.** 1982. Measurement of electron transport activity in soil . *Microb. Ecol.* **8**: 163 -168.

12. **Walsh**, C. Enzymatic reaction mechanisms. W.H. Freeman and Company, New York. 1979

13.**Yamanaka, T., Y. Fukumori, M. Numata, and T. Yamazaki.** 1988. The variety of molecular properties of bacterial cytochromes containing Heme a, p.39-46. *In* M. Brunori and B. Cance (ed), Cytochrome oxidase: Structure, function, and physiopathology. New York Academy of Sciences, New York.

14. **Zimmerman R., R. Iturriaga, and J. Becker-Birick.** 1978. Simultaneous determination fo the total number of aquatic bacteria and the number thereof involved in respiration. *Appl. Env. Micro.*, **36**: 926-935.

CHAPTER 6

THE USE OF VEGETABLE OILS FOR THE ENGINEERED APPLICATION OF *PSEUDOMONAS STUTZERI* KC

The CO_2 trapping technique described in these studies was performed with the assistance of Dr. Michael Dybas

ABSTRACT

Vegetable oil may serve as a low cost non-toxic electron donor that could provide a controlled release of growth substrate to microorganisms for extended time periods. *Pseudomonas stutzeri* KC was tested for its ability to grow and transform carbon tetrachloride(CCl_4) when vegetable oils are used as the carbon and energy source. *P. stutzeri* KC achieved growth yields on vegetable oils under denitrifying conditions that were similar to those observed when acetate was the carbon and energy source. ^{14}C-CCl_4 studies revealed that 40 - 50% of the originally added CCl_4 was converted to CO_2. Corn oil was used to evaluate the long term growth characteristics and CCl_4 transformation capacity of *P. stutzeri* KC and of an enrichment derived from CCl_4 contaminated aquifer solids from Schoolcraft, MI. CCl_4 masses of 18.3 ± 0.8 and 17.2 ± 1.3 μg were removed by *P. stutzeri* KC and *P. stutzeri* KC combined with the Schoolcraft flora respectively. No significant CCl_4 transformation was observed for the Schoolcraft flora alone. Approximately 5% of the initially added CCl_4 was converted to chloroform by *P. stutzeri* KC. Growth curves, pH, and nitrate analysis revealed that the yield and rates of growth were limited by the addition of nitrate.

INTRODUCTION

Three hundred to four hundred thousand subsurface hazardous waste sites have been identified which impact groundwater [7]. A majority of these sites contain various levels of toxic organics, and many current remediation technologies are designed to treat these wastes [3]. In addition, many groundwaters are contaminated with high levels of nitrate from agricultural operations. Nitrate contamination can render groundwater unusable as drinking water. Thus, remediation technologies that remove both nitrate and organic contamination are desirable.

Bioremediation, the use of microorganisms (either native or introduced) to degrade contaminants to harmless endproducts and biomass, is a promising approach for environmental cleanup. Many highly chlorinated contaminants are only biodegraded by cometabolism, which is defined as the fortuitous transformation that depends on the previous or concurrent utilization of a growth substrate[1]. By virtue of their oxidized state, these highly chlorinated organic solvents are poor energy sources. Growth substrates (electron donors) must be provided to allow for the growth of cometabolic populations and to sustain the cometabolic reaction. A major factor limiting cometabolism is delivery of the growth substrate needed to sustain a biomass capable of degrading the target contaminant. Of primary concern is the cost associated with repeated delivery and mixing of nutrients in subsurface environments. Use of a low cost electron donor that does not need to be delivered repeatedly would be highly desired.

In this chapter, I report on the growth of *P. stutzeri* KC on vegetable oils and its subsequent cometabolism of CCl_4 with nitrate as the electron acceptor. Additionally, I report on the transformation capacity of *P. stutzeri* KC and the organisms long term growth characteristics using vegetable oil as a growth substrate.

MATERIALS AND METHODS

Organisms. *P. stutzeri* KC (DSM deposit no. 7136, ATTC deposit no. 55595), derived originally from aquifer solids from Seal Beach, CA, [2] is routinely maintained in our laboratories on nutrient agar plates. An enrichment of groundwater microorganisms was obtained from CCl_4-contaminated aquifer solids from Schoolcraft, MI. The enrichment was obtained by incubating 1 g of aquifer solids in 100 mL of SGW media at pH 8.2 under denitrifying conditions at 20-23 °C. The enrichment was used as an inoculum in CCl_4 transformation experiments after protein levels reached 89 ± 12 mg/L.

Chemicals and radioisotopes. All chemicals used were ACS reagent grade (Aldrich or Sigma Chemical Co.). All water used in reagent preparation was 18 Mohm resistance or greater. CCl_4 (99% -purity) was obtained from Aldrich Chemical Co., Milwaukee, Wis. ^{14}C-labeled CCl_4 (3.4 mCi/mMol) was obtained from NEN DuPont (Boston, MA).

Media. Hanford simulated groundwater (SGW) was identically prepared as described in Chapter 3, with the exception that acetate was not added to the growth medium. After pH adjustment, media was transferred to growth vials and supplemented with vegetable oils as the electron donor source. Medium D was prepared as described in Chapter 2.

Initial screening of CCl_4 transformation activity for *P. stutzeri* KC cultures grown on vegetable oils. *P. stutzeri* KC was screened for growth and transformation of CCl_4 on corn oil, soybean oil, and canola oil. All three types of oil were obtained from a local food market in Lansing, MI. One liter of SGW medium (acetate free) was prepared at pH 8.2. Fifteen milliliters of medium were dispensed into 30 mL serum bottles (Wheaton), and 50 μL of oil was dispensed into test vials receiving oil. Serum bottles were degassed through the interlock of an anaerobic glove box, (Coy laboratories, Ann Arbor, MI) and

sealed under anoxic conditions with 20 mm Teflon-lined butyl rubber septa (West) and aluminum crimp seals (West no. 5120-1180). Samples were then autoclaved at 121°C for 20 minutes. Following autoclaving, Tween 80 (polyethylenesorbital monooleate) was added as an emulsifier to half of the samples at a final concentration of 0.01% (v/v). A mass of 2.1 ± 0.7 μg of ^{14}C-CCl_4 was added to all vials prior to inoculation. *P. stutzeri* KC was added as a 1% inoculum (v/v) from a 48 hour medium D grown culture that was washed and resuspended to its original volume (100 mL) in 50 mM phosphate buffer at pH 8.2. Abiotic controls received CCl_4 but no organisms. Cultures were grown for 72 hours before being assayed for CCl_4, ^{14}C-CO_2, and protein .

Due to the partitioning of CCl_4 into the oil phase, the 30 mL serum bottles from the initial screening experiment were heated at 90 °C for 2 hours prior to GC analysis. Bottles removed from the water bath were immediately assayed by removing 100 μL of headspace gas with a 500 μL Pressure-Lok® gas syringe (Supelco) and injecting the sample into a gas chromatograph as described in Chapter 2. External calibration curves were prepared by addition of a primary standard (8.37 ng of CCl_4 per μL of methanol) to secondary aqueous solutions having the same gas/water ratio, water/oil ratio, ionic strength, incubation temperature, and speed of shaking as the assay samples. A five point calibration curve was prepared over a concentration range bracketing that of the assay samples.

Protein was assayed by the modified Lowry method, with bovine serum albumin as the standard [6].

^{14}C-CO_2 determination. Following GC analysis, both *P. stutzeri* KC cultures and controls were acidified with 300 μL of 6 N HCl (final pH of 2). N_2 gas (100 mL) was flushed through the headspace of the vials and transferred via teflon lines to 1 mL of 3N

KOH in 2 mL serum vials sealed with 11 mm teflon lined aluminum seals. N_2 gas (100 mL) was then flushed through the base trap to remove any residual CCl_4. The ratio of transfer gas to vial headspace was 10:1, and the ratio of stripping gas to headspace was 100:1 in the base trap. Base (100 μL) was added to 10 mL of scintillation cocktail (Beckman). All samples were counted for 5 minutes on a Packard tri-carb liquid scintillation counter (Model 1500).

Transformation capacity and long term growth characteristics. Corn oil was chosen as the growth substrate in an experiment to determine the long term growth characteristics and transformation capacity of oil grown cultures. One liter of SGW medium (acetate free) was prepared at pH 8.2 with 2X phosphate buffer (27.22 g/L KH_2PO_4). The 2X phosphate concentration increased the buffering capacity in order to prevent the strong alkaline conditions that frequently arise during growth under denitrifying conditions. Ten milliliters of medium were dispensed into 20 mL automated headspace sampler vials (Alltech), and 50 μL of corn oil was added to each vial. Vials were degassed through the interlock of an anaerobic glove box and sealed under anoxic conditions using teflon lined butyl rubber septa and aluminum crimp seals. Vials were autoclaved at 121°C for 20 min. CCl_4 was added from a sterile aqueous phase stock at a final mass of 9.5 ± 0.6 μg. *P. stutzeri* KC was added as a 1% inoculum (v/v) from a 72 hour culture grown in SGW medium with corn oil as the electron donor under denitrifying conditions. The initial protein level for samples receiving *P. stutzeri* KC was approximately 12 mg/L (6.9×10^7 cells/mL). The Schoolcraft samples received a 1% inoculum (v/v) from an enrichment of aquifer solids as described in the organisms section of materials and methods. Schoolcraft samples had an initial protein concentration of approximately 0.9 mg/L. Samples labeled P. KC + Schoolcraft received a 1% inoculum (v/v) of each culture. The abiotic controls received CCl_4 but no organisms. On days indicated in the figures, 3 vials from each set were assayed for CCl_4, protein, and nitrate.

CCl_4 and chloroform were analyzed on a Perkin Elmer Auto System equipped with a PE 624 - 0.533 diameter - 50 meter column (PE No. N9312844) and an electron capture detector with nitrogen carrier (30 psi) and helium make-up gas. The GC had a cycle time of 5.0 minutes, an oven temperature of 80°C, and detector and injector temperatures of 250°C. The GC was connected to a Perkin Elmer Model HS-40 automated headspace sampler. The autosampler had a thermostating temperature of 90 °C, a needle temperature of 120 °C, a thermostating time of 12 minutes, a pressurization time of 2.0 seconds, and an injection time of 0.15 minutes. The headspace sampler had nitrogen carrier gas with a pressure of 30 psi. External calibration curves were prepared by addition of a primary standard (0.44 μg of CCl_4 per μL of methanol and 0.2 μg of chloroform per μL of methanol) to secondary aqueous solutions having the same gas/water ratio, water/oil ratio, ionic strength, and incubation temperature as that of the assay samples. An eight point calibration curve was prepared over a concentration range bracketing that of the assay samples.

Aqueous phase samples were filtered through 0.22 μm nylon Titon® syringe filters and diluted 1:10 prior to analysis for nitrate. Nitrate concentrations were determined by capillary electrophoresis with indirect photometric detection (CE-IPD) using a model 270 capillary electrophoresis system (Applied Biosystems Inc.) equipped with a 72 cm x 50 μm capillary column micro-coated for anion analysis with a 2% ethylene glycol solution. The detection electrode was anodic using 30 kV of applied voltage. The capillary electrophoresis system was interfaced to a PC via a 900 Interface with data acquired within Turbochrome (PE-Nelson) chromatography processing software. A five point calibration curve was prepared over a concentration range bracketing that of the assay samples and was analyzed by the same method as that of the test samples.

RESULTS

Initial screening of CCl_4 transformation activity for *P. stutzeri* KC cultures grown on vegetable oils. Transformation of CCl_4 by *P. stutzeri* KC generates CO_2, a cell associated product(s), and a non-volatile product(s) [2]. Table 6.1 illustrates that corn, soybean, and canola oil were all suitable growth substrates for *P. stutzeri* KC, with growth yields similar to those observed in Medium D with acetate as the growth substrate. An emulsifier, Tween 80, was not necessary to facilitate the growth of the organism. All of the originally added CCl_4 was transformed by the organism during the 72 hour growth period. ^{14}C-CO_2 measurements showed that approximately 40 - 50% of the original CCl_4 was converted to CO_2. The CO_2 values for the samples containing Tween 80 were approximately five times lower, suggesting that Tween 80 affects the pathway of transformation.

Table 6.1. Growth of *P. stutzeri* KC on various vegetable oils and subsequent CCl_4 transformation.

Sample	Protein Yield mg/L	Mass CCl_4 Remaining μg^a	^{14}C - CO_2 CPM[d]
Abiotic Control, Soybean Oil	14±12[a]	2.1±0.7	0
Abiotic Control, Soybean Oil and 0.01% Tween 80[b]	5±6	1.8±0.1	0
Abiotic Control, No Oil Added	11±5	1.7±0.3	0
Abiotic Control, No Oil and 0.01% Tween 80	5±7	1.8±0.2	0
P. KC in Medium D	291±37	ND[c]	ND
P. KC and Corn Oil	264±17	0.0±0.0	14,681±3,878
P. KC, Corn Oil and 0.01% Tween 80	306±32	0.1±0.0	3,941±559
P. KC and Soybean Oil	237±19	0.0±0.0	21,404±4,751
P. KC, Soybean Oil and 0.01% Tween 80	260±1	0.2±0.2	4,077±1,022
P. KC and Canola Oil	270±15	0.0±0.0	15,727±3,070
P. KC, Canola Oil, and 0.01% Tween 80	228±28	0.0±0.0	7,036±283

a. Mass of CCl_4 added was 2.0 μg

b. Tween 80 (Poloxethylenesorbitan Monooleate) was added as an emulsifier

c. CCl_4 transformation was not monitored for the *P. stutzeri* KC that was grown in Medium D.

d. ^{14}C - CCl_4 had a specific activity of 3.4 mCi/mmole

Transformation capacity and long term growth characteristics. Corn oil was evaluated as a long term growth and energy substrate using both *P. stutzeri* KC and a consortium of groundwater microorganisms obtained from an enrichment of aquifer solids from a CCl_4-contaminated aquifer in Schoolcraft, MI. Figure 6.1 illustrates the cumulative mass of CCl_4 transformed by cultures of *P. stutzeri* KC over 13 days. The mass of CCl_4 removed was equivalent to a concentration of 1.83 ± 0.08 mg/L. Figure 6.1 also indicated chloroform was produced by cultures of *P. stutzeri* KC grown on corn oil. The final mass of chloroform produced was approximately 5% of the originally added CCl_4 mass. Figure 6.2 illustrates the mass of CCl_4 removed by the Schoolcraft consortium. When compared to the abiotic loss of CCl_4 (Figure 6.3), it cannot be concluded that the Schoolcraft consortium achieved significant removal of CCl_4. The Schoolcraft consortium removed 4.59 ± 0.75 μg of CCl_4 while the abiotic losses were 4.13 ± 0.61 μg of CCl_4. Although difficult to observe on Figure 6.2, chloroform (0.08 ± 0.04 μg) was observed in the Schoolcraft consortium samples. Figure 6.4 shows the mass of CCl_4 removed and chloroform produced by a mixture of *P. stutzeri* KC and Schoolcraft consortium. *P. stutzeri* KC was added to Schoolcraft consortium at a ratio of 12:1 based on initial protein levels. The similarity of Figure 6.4 to Figure 6.1 indicates that CCl_4 removal and chloroform production were attributable to *P. stutzeri* KC.

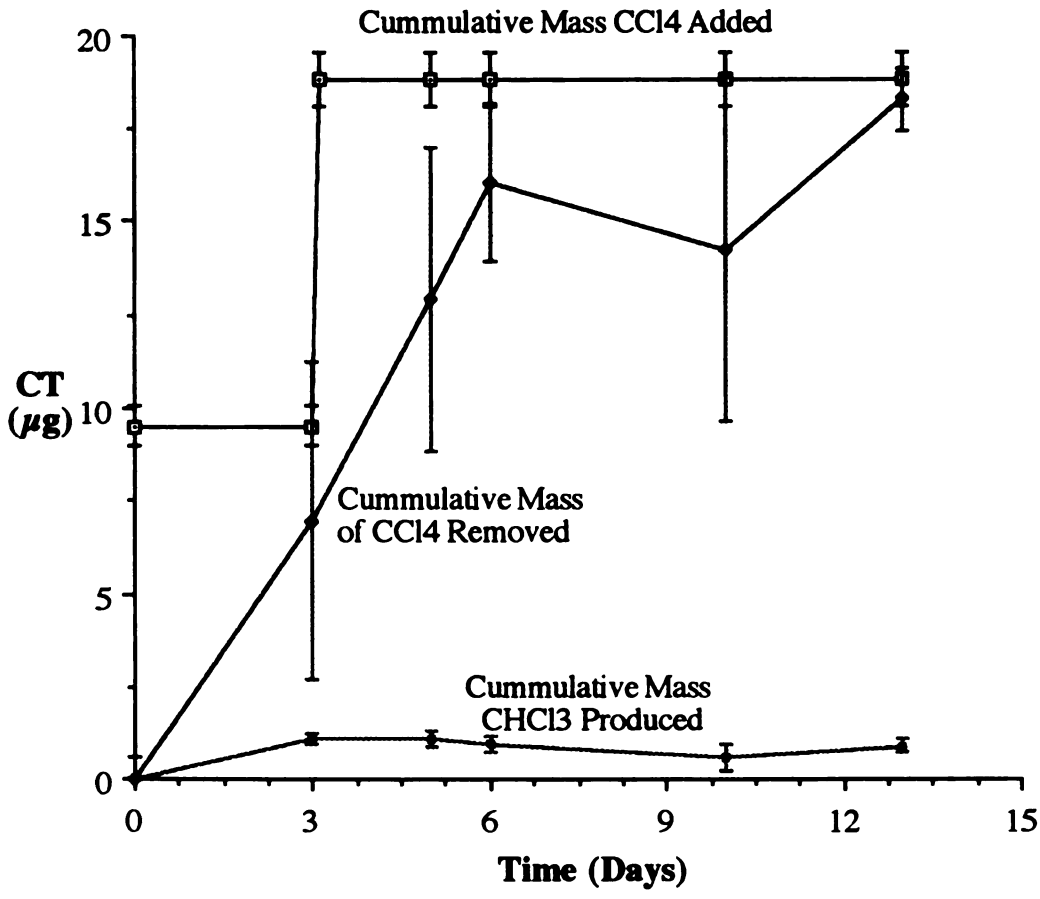

Figure 6.1. The cumulative mass of CCl_4 removed and chloroform produced by *P. stutzeri* KC. CCl_4 was added initially and on day 3. Error bars for the mass of CCl_4 added represent the cumulative error of addition. Error bars for experimental samples represent a standard deviation of three independently grown cultures.

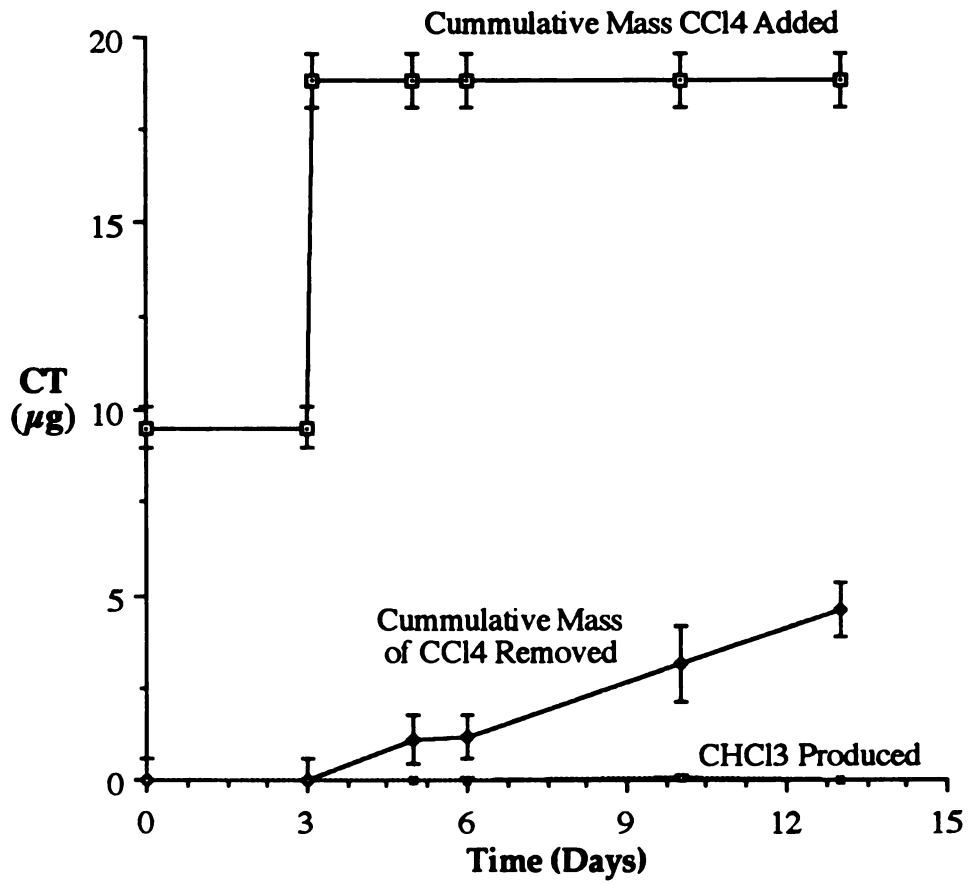

Figure 6.2. The cumulative mass of CCl_4 removed and chloroform produced by an enrichment of organisms from Schoolcraft aquifer solids. CCl_4 was added initially and on day 3. Error bars for the mass of CCl_4 added represent the cumulative error of addition. Error bars for experimental samples represent a standard deviation of three independently grown cultures.

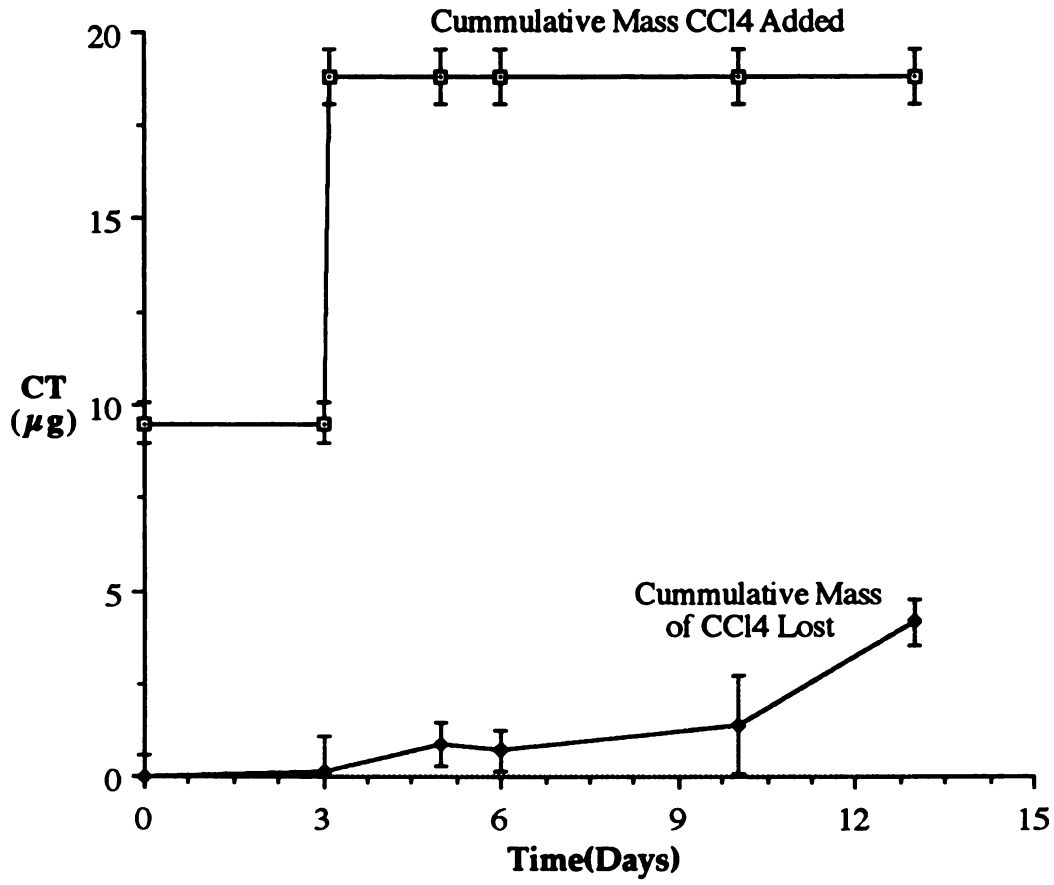

Figure 6.3. Abiotic loss of CCl_4. Error bars represent the standard deviation of 3 independent control samples. CCl_4 was added initially and on day 3. Error bars for the mass of CCl_4 added represent the cumulative error of addition. Error bars for experimental samples represent a standard deviation of three independently grown cultures.

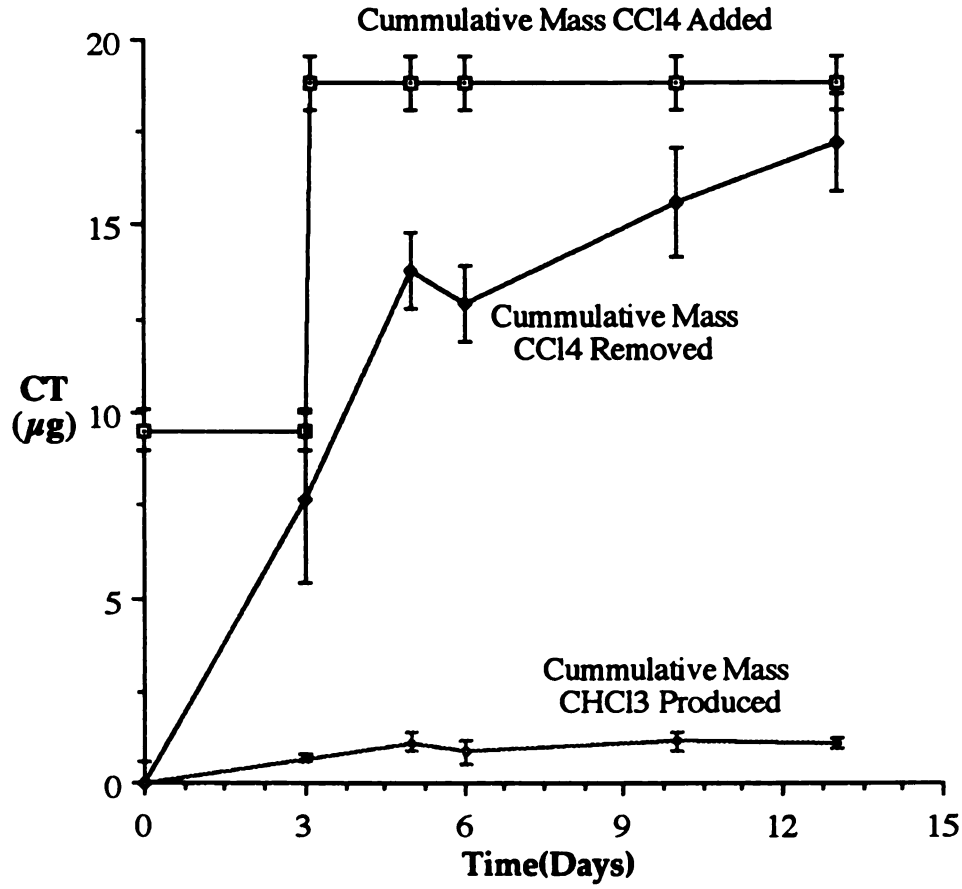

Figure 6.4. The cumulative mass of CCl_4 removed and chloroform produced by a mixture of *P. stutzeri* KC and an enrichment of organisms from Schoolcraft aquifer solids. CCl_4 was added initially and on day 3. Error bars for the mass of CCl_4 added represent the cumulative error of addition. Error bars for experimental samples represent a standard deviation of three independently grown cultures.

Figure 6.5 shows the growth of *P. stutzeri* KC, Schoolcraft consortium, and *P. stutzeri* KC + Schoolcraft consortium on corn oil under denitrifying conditions. Figure 6.6 illustrates the concentrations of nitrate remaining in the three test conditions over the time of the study. In viewing the two figures together, it becomes apparent that growth stopped after the nitrate was consumed. On day 12, a final nitrate concentration of approximately 62 mg/L was added to the samples, which may account for the late increase in growth observed for the *P. stutzeri* KC + Schoolcraft samples. The final pH

of the samples did not exceed 8.42 ± 0.11, and it should be noted that visible oil still remained in the sample vials. Therefore, I concluded that nitrate was the growth limiting factor in the experiment.

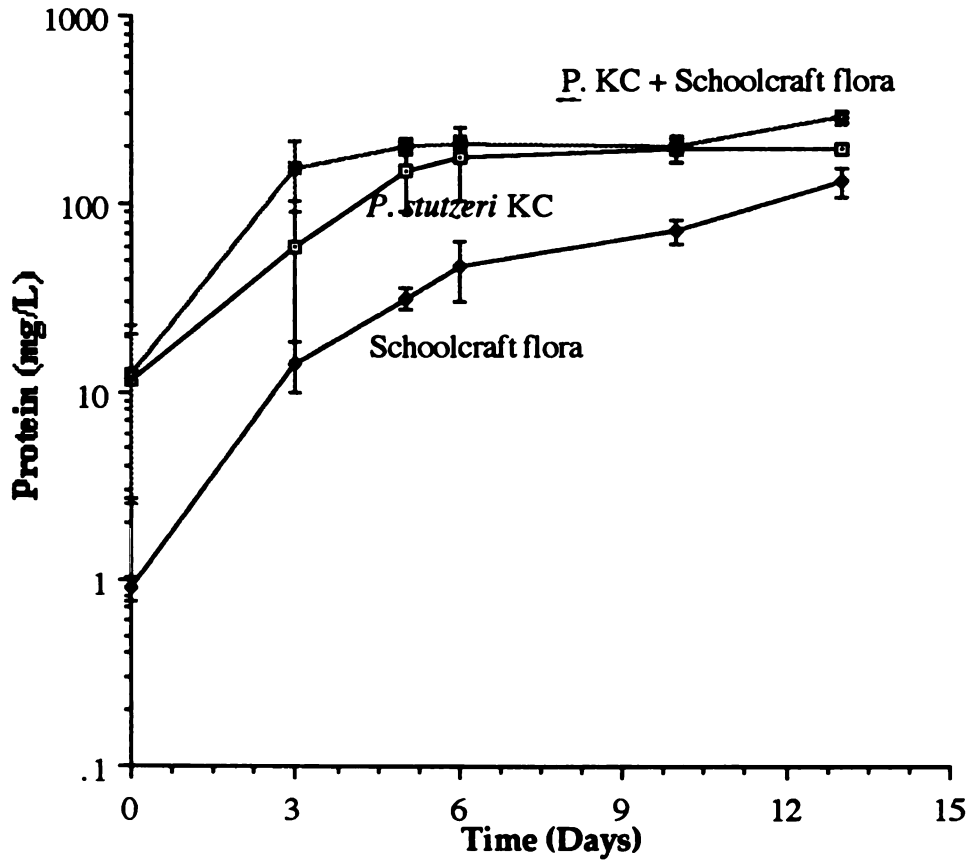

Figure 6.5. Growth of *P. stutzeri* KC, Schoolcraft consortium, and *P. stutzeri* KC + Schoolcraft consortium. Error bars represent the standard deviation of 3 independently grown cultures.

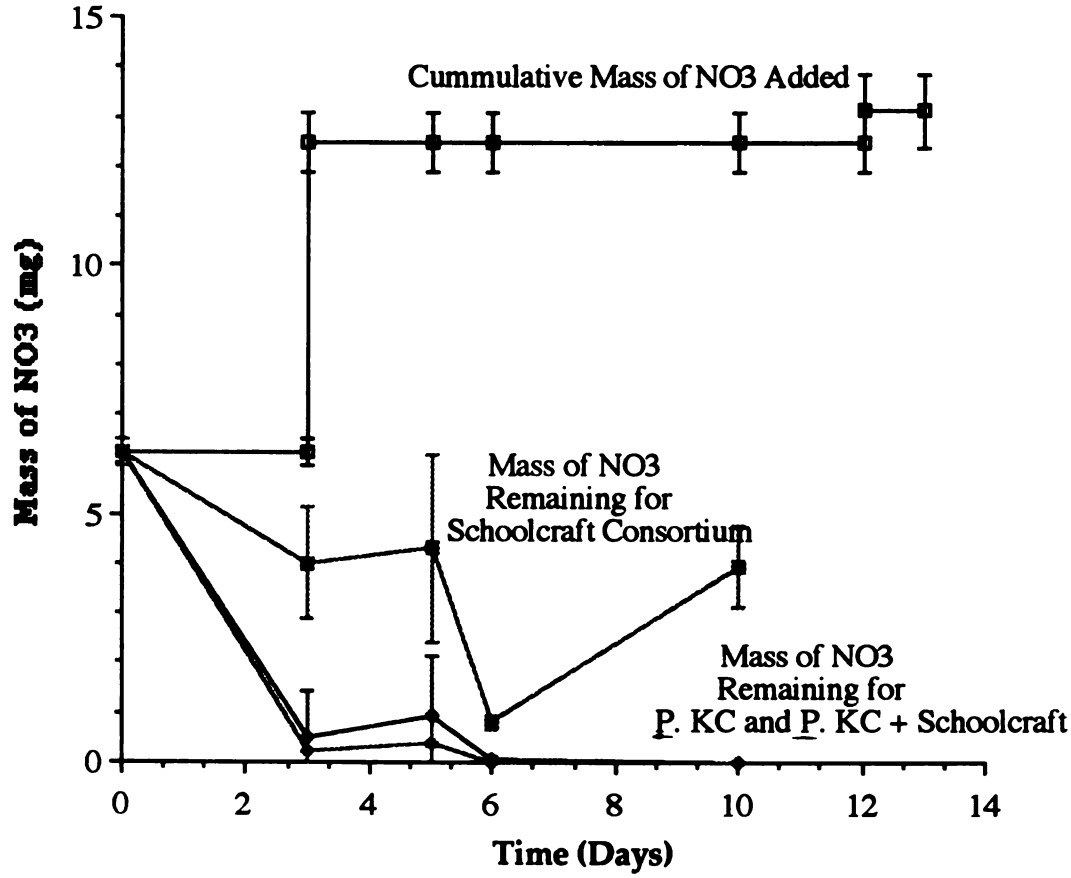

Figure 6.6. Mass of NO_3 remaining in cultures of *P. stutzeri* KC, Schoolcraft consortia, and *P. stutzeri* KC + Schoolcraft consortia. Error bars represent the standard deviation of 3 independent cultures.

DISCUSSION

The present work provides a favorable outlook for the application of vegetable oils as growth and carbon sources for in situ bioremediation. Table 6.1 illustrates that *P. stutzeri* KC is capable of growth on various vegetable oils and subsequent transformation of CCl_4. The addition of an emulsifier, Tween 80, was not required to aid the organisms is breaking down the long chain fatty acids that comprise vegetable oils. A conversion of 40 -50% of the originally added CCl_4 to ^{14}C-CO_2 indicates that the transformation

pathway for oil grown cells is very similar to that seen for cells grown on acetate. The lower values for CO_2 observed for cells grown in the presence of Tween 80 suggests that the emulsifier alters the pathway of transformation. Lewis and Crawford [4] reported that amount of organics present in the culture medium can greatly influence product distribution, with high levels of organics lowering the percentage of CCl_4 that is converted to CO_2.

The cumulative mass of CCl_4 removed by *P. stutzeri* KC (Figure 6.1) in the long term growth experiments is very similar to the 24 hour transformation capacity reported by Tatara et al [8] for *P. stutzeri* KC grown in medium D. As Figures 6.1 and 6.5 illustrate, the cultures continued to transform CCl_4 and grow over the 13 day time period, with rates influenced by NO_3^- concentrations (Figure 6.6). These results indicate the advantage that oil presents over acetate as a growth substrate for in situ bioremediation. Higher growth rates are typically observed with acetate grown cultures. *P. stutzeri* KC will typically reach stationary phase within 72 hours following inoculation in Medium D [8]. Using oil as a growth substrate, organisms are limited by the solubility of oil in water, and by the supply of electron acceptors (NO_3^-) and other essential nutrients.

To the best of my knowledge, this is only the second report of *P. stutzeri* KC producing appreciable chloroform as a product of CCl_4 transformation (Figures 6.1 and 6.4) Lewis and Crawford [5], reported that approximately 5% of the originally added CCl_4 was converted to chloroform in a HEPES buffered medium under denitrifying growth conditions. They also [4] reported that medium composition and levels of organics can greatly influence end-products of CCl_4 transformation and also influence the distribution of these products. In this study, the mass of chloroform produced was also approximately 5% of the mass of CCl_4 added, and the presence of oil may also have influenced this product formation. Figure 6.7 illustrates the pathway for chloroform production in the

presence of lipids [Criddle, Ph. D. Thesis]. The trichloromethyl radical is capable of abstracting hydrogen from lipids to form chloroform. Therefore, if a tri-chloromethyl radical is produced during the transformation of CCl_4 by *P. stutzeri* KC, then the presence of oil may explain the production of low levels of chloroform. Alternatively, since this is the first observed production of chloroform seen in our laboratory, method changes must also be considered as well. It is possible that chloroform production was not detected previously because I typically performed assays with much lower CCl_4 concentrations. At CCl_4 concentrations of 20 μg/L, the conversion of 5% of the initially added CCl_4 to chloroform would result in chloroform levels below detection limits (method detection limit of 2 μg/L). The new GC-protocol developed for this experiment has increased sensitivity to chloroform (method detection limit of 0.5 μg/L). In addition, approximately 50-fold higher concentrations of chloroform were utilized in this set of experiments, therefore chloroform was easily detected at the concentrations produced. Despite the varying explanations for the observation of chloroform as an end-product of CCl_4 transformation, this work warrants further pursuit as the growth of *P. stutzeri* KC on oil may provide insight on the pathway of CCl_4 transformation.

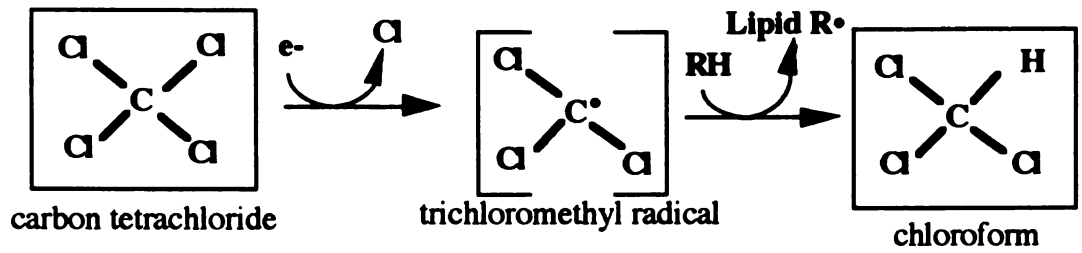

Figure 6.7. Production of chloroform from the reaction of a trichloromethyl radical with a lipid molecule. Adapted from Criddle, Ph.D. Thesis [1a].

The growth of *P. stutzeri* KC and transformation of CCl_4 in the presence of microorganisms enriched from aquifer material is also promising for field application (Figure 6.4). Little or no transformation was observed for Schoolcraft consortium (Figure 6.2 and 6.3) despite the fact that the consortium grew very well with oil as a growth

substrate (Figure 6.5). This indicates that *P. stutzeri* KC is able to successfully compete with groundwater bacteria when KC is provided with an inoculum advantage and the pH is optimum.

The use of vegetable oil as a growth substrate could enable *P. stutzeri* KC to transform higher levels of CCl_4 than previously reported. Concentrations of CCl_4 higher than 5 mg/L can be toxic to *P. stutzeri* KC (data not shown). In an oil/water system, CCl_4 will tend to partition into the oil phase. This would tend to reduce aqueous phase concentrations of CCl_4. For a given total mass of CCl_4 in the system, organisms experience lower aqueous phase concentrations of CCl_4. Rates of transformation would therefore be dependent upon equilibrium chemistry and diffusion of secreted factor and/or CCl_4 out of the oil phase. These physical and chemical properties may enable biological removal of CCl_4 in subsurface environments where CCl_4 concentrations were previously considered excessive. However, delivery of vegetable oils to the subsurface environment poses a major engineering challenge. The hydrophobic nature of oil and its viscosity make it difficult to evenly disperse in the subsurface. Future studies need to be conducted on methods of oil introduction. Column and model aquifer studies should be performed to further asses the feasibility of oil as a long term growth substrate for *in* situ bioremediation.

REFERENCES

1.**Criddle, C.S.** 1993. The kinetics of cometabolism. *Biotech. Bioeng*. **41:** 1048 - 1056.

1a. .**Criddle, C.S.** 1989. Ph.D. disseratation. Stanford Universtiy, Stanford, Calif.

2.. **Criddle, C. S., J. T. DeWitt, D. Grbić-Galić, and P. L. McCarty.** 1990. Transformation of carbon tetrachloride by *Pseudomonas* sp. strain KC under denitrification conditions. *Appl. Environ. Microbiol.*. **56**:3240-3246.

3. **Fredrickson, et. al.** 1993. Enhancement of *in-situ* microbial remediation of aquifers. United States Patent. Patent Number: 5,265,674.

4. **Lewis, T.A. and R.L. Craword.** 1995. Transformation of Carbon tetrachloride via sulfur and oxygen substitution by *Pseudomonas* sp. strain KC. *J. Bact.* **177:** 2204-2208.

5. **Lewis, T. A., and R. L. Crawford.** 1993. Physiological factors affecting carbon tetrachloride dehalogenation by the denitrifying bacterium *Pseudomonas* sp. strain KC. *Appl. Environ. Microbiol.* **59**:1635-1641.

6. **Markwell, M. A., S. M. Haas, N. E. Tolbert, and L. L. Bieber.** 1981. Protein Determination in membrane lipoprotein samples: manual and automated procedures. *Methods Enzymology*. **72**:296-301.

7. **Office of Emergency and Remedial Response.** 1993. Evaluation of the likelihood of DNAPL presence at NPL sites. EPA Report 540-R-93-073 PB93-963343)

8. **Tatara, G.M., M.J. Dybas, and C.S. Criddle.** 1993. Effects of medium and trace metals on kinetics of carbon tetrachloride transformation by *Pseudomonas* sp. strain KC. *Appl. Environ. Microbiol.* **59**:2126-2131.

CHAPTER 7

CONCLUSIONS AND FUTURE INVESTIGATIONS

The main goal at the outset of this study was to elucidate the biochemical components and processes responsible for CCl_4 degradation by *P. stutzeri* KC. Although several questions remain unanswered at the conclusion of my work, I have made significant progress in understanding many aspects of this interesting and complex system.

Preliminary studies performed on *P. stutzeri* KC focused on understanding how trace metals affected the kinetics of CCl_4 transformation. These studies were designed to examine an initial hypothesis proposed by Criddle et. al [1991], which stated that an iron scavenging system cometabolically transformed CCl_4. My initial work provided support for this hypothesis by determining that the cometabolic transformation of CCl_4 was dependent on the growth of *P. stutzeri* KC in iron limited and copper-containing medium. These preliminary investigations also established that addition of ferric iron to an actively-transforming-culture inhibited CCl_4 transformation.

Based on the hypothesis that the secreted factor was a siderophore, I hypothesized that other cell-types will be able to transform CCl_4 in the presence of the secreted factor. This hypothesis was based on previously published iron uptake studies which demonstrated that many species of microorganisms are able to use exogenously supplied siderophores from other cell-types to obtain iron. The finding that *P. fluorescens* was able to transform CCl_4 in the presence of the secreted factor from *P. stutzeri* led to the discovery that many diverse cell types, including gram positive and even eukaryotic organisms are capable of rapid CCl_4 transformation in the presence of the secreted factor. The ability of other cells to transform CCl_4 when combined with the secreted factor led to the development of a bioassay which was critical in the purification processes, and also provided a tool to perform many interesting physiological studies. Using the bioassay for the secreted factor, I was able to determine that aerobically-grown *P. stutzeri* KC secretes the factor, oxygen reversibly inhibits CCl_4 transformation, live cells are required for

activation of the factor, the factor is readily transported through aquifer material, the factor is stable indefinitely after lyophilization to powder, and the pH optimum for CCl_4 transformation is approximately 8.5.

To further test the iron scavenging hypothesis, I subjected culture supernatant to ultrafiltration, and combined the size fractionated supernatant with washed cells of *P. stutzeri* KC. This process established that both extracellular and intracellular factors were involved in the transformation. By themselves, washed cells of *P. stutzeri* KC did not transform CCl_4 to a significant degree. Occasionally, CCl_4 transformation observed in cell-free culture supernatant, but this activity was not reliable. Rapid and reliable CCl_4 transformation was only obtained when washed whole cells were combined with culture supernatant. Fractionation of culture supernatant established that the extracellular factor is small with an apparent molecular weight of 500 daltons. I also established that the inhibitory effects of iron are largely due to a supernatant factor with a molecular weight greater than 10,000 daltons. This data conflicted with the siderophore hypothesis, suggesting that elucidation of the normal physiological role of the CCl_4 transformation system might best be achieved by purification and identification of the secreted factor. Although this goal was not fully achieved, significant progress was made toward purification of the secreted factor. A procedure was developed following the discovery that the secreted transforming factor is stable after lyophilization to dryness, and is extractable with acetone. Additionally, a fraction containing transformation activity eluted at 27 - 28 minutes from a semi-preparative reverse phase HPLC column at a flowrate of 7 ml/min with a methanol/water gradient. However, this peak of activity contained several constituents as evaluated by mass spectrometry, thus further purification is required.

To localize the cell dependent transformation activity, I performed crude cell extract experiments with *P. stutzeri*. These studies established that CCl_4 transformation required a source of reducing equivalents (NADH), a cell membrane, and the secreted factor. This finding led me to hypothesize that the secreted factor accepted reducing equivalents for CCl_4 transformation from a respiratory chain enzyme. To test this hypothesis, I performed respiratory chain inhibitor studies. The results of these studies did not support my hypothesis, but did support the hypothesis that non-respiratory electron transport proteins might be important. This hypothesis was supported by an experiment in which *L. acidophilus*, an organism lacking any membrane bound cytochromes or other electron transfer enzymes did not significantly transform CCl_4 when combined with the secreted factor.

The above studies have aided in the development of engineering applications of *P. stutzeri* KC. Studies on the effects of trace metals, pH, and kinetics of CCl_4 transformation by *P. stutzeri* KC provided critical baseline information for a bioaugmentation experiment conducted in a CCl_4-contaminated aquifer in Schoolcraft, MI. Additionally, studies using vegetable oil as a slow release growth substrate may led to the development of novel technologies to more effectively remediate CCl_4 and other hazardous waste sites.

The attempt to determine the mechanism and pathway of CCl_4 transformation through elucidation of the biochemical components responsible for the transformation was not successful. However, I established that the production of CO_2 and a non-volatile fraction by *P. stutzeri* KC did not involve any interconversion of these products. This data refuted a previous hypothesis which stated that the production of CO_2 resulted from the oxidation of non-volatile product(s), and suggested instead that production of CO_2 and non-volatile products occurs via parallel pathways. This data also supported the conclusion that

formate was not further oxidized to CO_2. Therefore, the most acceptable current model would involve the production of phosgene and/or thiophosgene from a dichlorocarbene radical as proposed in Chapter 1. However, at this point it is not yet possible to discriminate between one- and two-electron reduction pathways to exist.

Below, I provide a speculative model for CCl_4 transformation by *P. stutzeri* KC, which is consistent with the known features of this transformation. The model posits that CCl_4 accepts electrons from a reduced form of the secreted factor produced during growth in medium that contains copper and is iron limited. The oxidized form of the secreted factor is capable of further transformations only after reduction at the cell membrane by a non-respiratory enzyme. Additional data in support of such a model are as follows: 1) cell-free transformation activity is occasionally observed, 2) changes in product distribution based on changes in the buffer composition of the medium were observed by Lewis and Crawford [1995], 3) low levels of cell-associated products are produced even though very reactive intermediates such as phosgene and thiophosgene are produced [Lewis and Crawford, 1995], 4) very low chloroform production is observed indicating that intermediates are not reacting with membrane associated lipids, and 5) iron inhibition is linked to a large molecular weight extracellular component.

As an alternative model to that of Figure 7.1, it might be argued that the secreted factor binds CCl_4 for subsequent transformation at the cell membrane, possibly by a ferrisiderophore reductase. Such a model deserves consideration, but does not appear consistent with the observation that cells of *P. stutzeri* strain EP3-071388 transform CCl_4 when combined with the secreted factor following growth in iron excess conditions, nor with the observation of occasional cell-free transformation activity. Furthermore, in studies performed by Lewis and Crawford, it was demonstrated that medium composition

greatly affects product distribution, thus providing further evidence that the transformation occurs extracellularly.

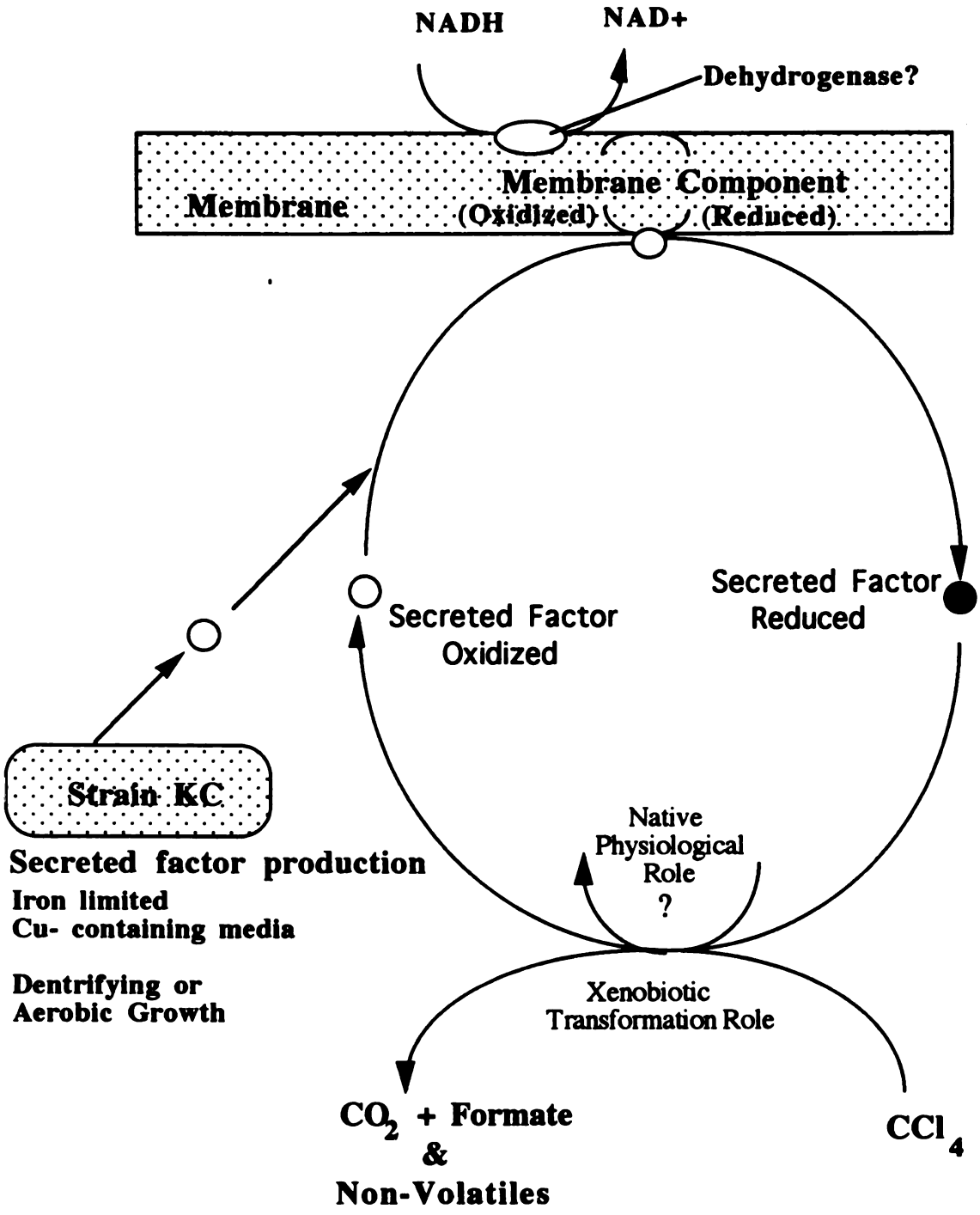

Figure 7.1 Model of proposed CCl_4 transformation process by *P. stutzeri* KC.

Future studies should focus on refining or refuting the model presented in Figure 7.1. The stoichiometry of the CCl_4 transformation reaction is needed to determine electron flux through the reaction(s). Using the crude membrane system, the electron balance should be determined for the oxidation of NADH, along with a chloride and carbon balance. These studies would determine the electron flux in the reaction so that conclusions can be drawn as to the flow of electrons in the reaction, as well as providing information to aid in elucidating the mechanism(s) for the transformation. Additionally, improvements in the purification protocol should be made by recombining fractions, altering wavelengths of absorbance for HPLC, changing the buffer in the eluent to possibly achieve better separation of the peaks, and possibly removing the filters from the purification procedure. Genetic analysis should focus on testing the secreted factor with ferrisiderophore reductase deficient mutants to determine if this enzyme is the membrane associated membrane responsible for transformation. These studies, may answer many of the questions that remain unanswered and will further clarify the molecular basis of this transformation.