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Transformation of Carbon Tetrachloride by *Pseudomonas* sp. Strain KC under Denitrification Conditions

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A denitrifying *Pseudomonas* sp. (strain KC) capable of transforming carbon tetrachloride (CT) was isolated from groundwater aquifer solids. Major products of the transformation of ^{14}C-labeled CT by *Pseudomonas* strain KC under denitrification conditions were $^{14}CO_2$ and an unidentified water-soluble fraction. Little or no chloroform was produced. Addition of dissolved trace metals, notably, ferrous iron and cobalt, to the growth medium appeared to enhance growth of *Pseudomonas* strain KC while inhibiting transformation of CT. It is hypothesized that transformation of CT by this organism is associated with the mechanism of trace-metal scavenging.

Microbial capabilities for dehalogenation are widely distributed in nature. This activity could potentially be exploited for in situ bioremediation of contaminated groundwater. The use of denitrifying organisms would be advantageous for aquifer bioremediation because, unlike oxygen, nitrate and nitrous oxide are highly soluble in water and easily added. Furthermore, nitrate is already present in many groundwaters because of the widespread use of fertilizers. Bouwer and McCarty (2) demonstrated that carbon tetrachloride (CT) and brominated trihalomethanes are transformed under mixed-culture denitrifying conditions. To date, however, there are no reports of pure-culture denitrifiers capable of haloaliphatic transformations. This has led some to speculate that nondenitrifying "secondary" organisms are responsible for biotransformations observed in mixed cultures under denitrification conditions (5).

To assess the possibility that subsurface denitrifiers might possess fortuitous dehalogenating capability, several denitrifying enrichments were prepared with aquifer solids as the source of microorganisms. Aquifer materials were obtained from sites in Seal Beach, Calif.; Moffett Field, Calif.; and Savannah River, Ga. These enrichments were screened for their activity toward CT. This screening culminated in the successful isolation of *Pseudomonas* sp. strain KC, an organism that rapidly and completely degrades CT, with carbon dioxide as its major end product. Several nondenitrifying organisms are also known to transform CT, but the products (chloroform and dichloromethane) are undesirable, and the rates of transformation can be slow. The metabolic basis for the transformation of CT by *Pseudomonas* strain KC was explored as this transformation may suggest a means for controlling reductive transformations in biological systems so as to produce innocuous products.

MATERIALS AND METHODS

Chemicals. CT (99+% pure) was obtained from Aldrich Chemical Co., Milwaukee, Wis. Chloroform (CF; Baker analyzed; Photrex grade 99.5% pure) was obtained from J. T. Baker Chemical Co., Phillipsburg, N.J. ^{14}C-labeled CT (250 μCi; 99% pure) with a specific activity of 4.3 mCi/mmol was obtained from NEN DuPont Research Products, Boston, Mass. Purity of the [^{14}C]CT and conditions of storage are described by Criddle et al. (4).

Medium preparation. All enrichments were grown in medium D, containing (per liter of degassed water) 2.0 g of KH_2PO_4, 3.5 g of K_2HPO_4, 1.0 g of $(NH_4)_2SO_4$, 0.5 g of $MgSO_4 \cdot 7H_2O$, 1 ml of trace nutrient stock TN2, 1 ml of 0.15 M $Ca(NO_3)_2$, 3.0 g of sodium acetate, and 2.0 g of sodium nitrate. Some experiments used different levels of acetate and nitrate. The pH of medium D was 7.0, but in the standard protocol it was adjusted to 8.0 with 3 N KOH before autoclaving. This adjustment caused a white precipitate to form, and a precipitate remained after autoclaving.

Trace nutrient 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 $B_3(OH)_3$, 0.12 g of NH_4VO_3, 1.01 g of $MnSO_4 \cdot H_2O$, and 1 ml of H_2SO_4 (concentrated). Trace nutrient stock solution TN3 contained (per liter) 3.0 g of $FeSO_4 \cdot 7H_2O$, 0.03 g of $Na_2MoO_4 \cdot 2H_2O$, 0.20 g of $ZnSO_4 \cdot 7H_2O$, 0.05 g of $Co(NO_3)_2 \cdot 6H_2O$, 0.02 g of $NiCl_2 \cdot 6H_2O$, 20 mg of $B_3(OH)_3$, and 25 mg of $MnSO_4 \cdot H_2O$.

Media were prepared in 1- or 2-liter flasks, degassed for 30 to 60 min under vacuum to remove traces of chloroform, and transferred to a Coy anaerobic glove box (Coy Laboratory Products, Ann Arbor, Mich.) for dispensing. The glove box had an atmosphere of 10% hydrogen–90% nitrogen. Initial enrichments were prepared in 160-ml serum bottles containing 100 ml of medium D and sealed with Mininert valves (individually pressure tested) equipped with a compression O ring to close off the throat of the serum bottle. The medium was then autoclaved for 20 min at 121°C, cooled, and transferred back to the glove box. A few grams of aquifer material was added, the bottles were resealed and removed from the glove box, and CT from a sterile stock solution was added to give a liquid phase concentration of 100 to 200 μg/liter. The side-port needles (Alltech catalog no. 943052) used for sampling the gas phase were autoclaved prior to use.

Enrichments and isolates. Materials used to obtain denitrifying enrichments were obtained from aquifer cores taken at

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Moffett Field, Calif. (courtesy G. Hopkins, Stanford University); and Savannah River, Ga. (courtesy J. Knezovich, Lawrence-Livermore National Laboratory); and from the Orange County Water District Well no. 7, Seal Beach, Calif., Naval Weapons Station (courtesy H. Ridgway, Orange County Water District).

Isolates were obtained from dilution series and by serial streaking on anaerobic and nutrient agar plates and on minimal medium plates (medium D plus 18 g of Noble agar per liter). Anaerobic plates were prepared with anaerobic thioglycolate agar, nutrient agar, and minimal media, and these plates were incubated under anaerobic conditions in a GasPak jar at 35°C. Other nutrient agar and minimal medium plates were incubated under aerobic conditions at room temperature. Morphologically distinct colonies were tested for their ability to degrade CT. Isolates were tested by transfer to a sealed bottle containing medium D, and 100 to 200 μg of CT per liter was added. Approximately 20 isolates were screened, and CT-degrading isolates were obtained only from the Seal Beach site. This site had no known previous exposure to CT. Nevertheless, transformation of CT was rapid (2 days), requiring no time for adaptation.

Biotransformation studies. Experiments with isolated organisms were conducted by dispensing 150 ml of media into 8-oz (250-ml) bottles with screw-cap tops (VWR catalog no. 16151-300) in the glove box and autoclaving. Two different bottle seals were used: septa and pressure-tested screw-cap Teflon Mininert valves. With septa, an open-hole screw cap (Pierce catalog no. 13219) was used over a layered combination of two septa: the bottom a Teflon-lined silicone septum (Alltech catalog no. 95322) and the top a Teflon-lined white rubber septum (Pierce catalog no. 12422). This combination of septa resisted deformation during autoclaving, had reasonably good resealability upon repeated penetration, and resisted loss of CT by sorption.

CT concentration in the gas phase of the test bottles was monitored by drawing 0.25 ml of gas phase into a 0.5- or 1.0-ml Precision gas-tight syringe (Alltech catalog no. 050032) outfitted with a side-port needle and injecting into a Tracor model MT-220 gas chromatograph equipped with a squalene packed column, a linearized electron capture detector, and a Spectra-Physics SP-4000 integrator. External standards were prepared by adding 150 ml of phosphate buffer (2.0 g of KH_2PO_4 per liter, 3.5 g of K_2HPO_4 per liter; pH 7.0) to a 250-ml bottle, stripping with nitrogen to remove residual CF, adding a primary standard of CT and CF in methanol (~0.56 μg of CT per μl of methanol, ~0.078 μg of CF per μl), equilibrating at sample incubation temperature on a shaker table, and analyzing on a gas chromatograph. The detection limit by this procedure was approximately 2 μg of CT per liter. Reported Henry's constants were confirmed for this media by using the EPICS procedure (9).

Samples of liquid culture were prepared (8) and analyzed for protein concentration by the method of Bradford (3), using bovine serum albumin as a standard.

Separation, identification, and quantification of ^{14}C-labeled compounds. Volatile gas components were separated by injection into a Packard 437A gas chromatograph operated isothermally at 165°C and equipped with an electron capture detector (260°C), an SGE splitter valve on the column effluent, and a model 561 compact flow unit. The packed column was Carbopack B (3% SP-1500 on 80/120 mesh; column length, 3 ft [ca. 91 cm], by 0.25-in. [ca. 0.64-cm] outside diameter). The pressure was set at 300 kPa for the helium carrier at the column entrance and 85 kPa for the argon-methane lines to the electron capture detector, providing 60 ml/min to the sample collection rack and 45 to 50 ml/min to the electron capture detector.

The SGE splitter valve was adjusted to divert nearly all (>99%) of the flow to a sample collection rack for trapping of individual components separated on the column. A very small (<1%) flow mixed with the argon-methane flowing into an electron capture detector. An eight-port programmed rotary valve directed flow for trapping in scintillation fluid to one of seven positions in the sample collection rack. Typical trapping intervals for compounds of interest were: 0 to 1.0 min for carbon dioxide; 1.0 to 2.2 min for dichloromethane; 2.2 to 6.1 min for chloroform; 6.1 to 9.1 min for carbon tetrachloride; and 6.1 to 12.4 min for late eluting unknowns. The trapping interval for carbon dioxide was confirmed by injecting $^{14}CO_2$ into the gas chromatograph and trapping the various gas fractions.

Liquid scintillation fluid (9 to 10 ml) was added to vials, which were attached to the sample collection rack. Scintillation cocktail for carbon dioxide gas received 1 ml of phenethylamine and 1 ml of methanol, giving a trapping efficiency of 99 to 104%. Cocktail for trapping CT was supplemented with 1 ml of isooctane. No supplements were added for other components. After trapping, vials containing the trapped fractions were removed from the rack, sealed, and counted on a liquid scintillation counter.

To quantify ^{14}C in liquid samples, a 4-ml culture sample was divided among three treatments: 1.0 ml was injected into a vial containing 0.15 ml of 1 N NaOH and degassed with nitrogen (fraction A); 1.0 ml was injected directly into a liquid scintillation cocktail amended with 1.0 ml of phenethylamine (fraction B); and 1.0 ml was filtered through a 0.2-μm-pore-size nylon filter into a vial containing 0.15 ml of 1 N HCl and degassed with nitrogen (fraction C). Fraction A included ^{14}C in cells, nonvolatile components, and carbon dioxide; fraction B contained total liquid sample ^{14}C; and fraction C contained nonvolatile components only. Degassing was accomplished by sonication of the liquid sample with a Bransonic 12 sonicator (Bransonic Cleaning Equipment Co., Shelton, Conn.) while passing a stream of nitrogen directed over the sample surface for 1 h. This method prevented foaming and removed 98% of the [^{14}C]CT while retaining 100% of the $^{14}CO_2$.

Cell ^{14}C was determined by centrifuging at 3,000 rpm in an IEC DPR 6000 centrifuge equipped with a model 850 rotor, washing with phosphate buffer, recentrifuging the pellet two more times at 4,000 × *g* with a bench top centrifuge, and digesting the washed pellet with 1.0 ml of 1 N hyamine hydroxide at 35°C until clear, as recommended by Botta et al. (1). Radioactivity associated with the cell material was operationally defined as either "bound" or "sorbed," depending on whether or not it could be removed by two washes with phosphate buffer: The sum of the sorbed and bound ^{14}C was subtracted from the counts for fraction A to obtain the counts associated with nonvolatile components plus carbon dioxide. Counts associated with fraction C were subtracted from this value to obtain the counts associated with dissolved carbon dioxide alone. Percent recovery was determined by adding each of the gas and liquid component fractions and dividing by the combined total counts for the gas and liquid. This value ranged between 84 and 100%.

All radioactivity was assayed on a Tri-Carb model 4530 scintillation spectrometer (Packard Instruments Co., Downers Grove, Ill.), and quench was corrected by the external standard channels ratio method (10).

TABLE 1. Characteristics of *Pseudomonas* sp. strain KC and *P. stutzeri*[a]

Organism	Cell length (μm)	Cell diam (μm)	Test																							
			Nitrate reductase	Phenylalanine	Citrate	Urea	Lysine	Arginine	Ornithine	Sucrose	Malonate	Anaerobic glucose	Adonitol	Aerobic glucose	Maltose	Arabinose	Inositol	Raffinose	Sorbitol	Lactose	Rhamnose	Growth with nitrite	Oxidase	Xylose	Glycerol
Pseudomonas strain KC	1.2–2.1	0.4–0.6	+	−	+	−	−	−	−	−	+	−	−	+[a]	−	−	−	−	−[b]	−	−	+[c]	+	−	+
P. stutzeri	1.4–2.8	0.7–0.8	+	d	+	NI	−	−	−	−	d	−	−	+	+	−	−	NI	−	−	−	+	+	−	+

[a] From *Bergey's Manual of Systematic Bacteriology* (12). d, 11 to 89% of strains are positive; NI, not indicated.
[b] + after 48 hs.
[c] Gas evolution observed.

RESULTS

Enrichments and isolation of *Pseudomonas* sp. strain KC. Enrichments differed in their response to CT for each of the sites examined. CT disappeared slowly in the enrichment sample from Moffett Field, Calif. At the Savannah River site, a variable response was observed for each depth examined: in the 21-m depth enrichment, CT disappeared very slowly; in the 24-m depth enrichment, CT disappeared in a 10-day period with concomitant CF production; in the 35-m depth enrichment, slow transformation was again observed with significant CF production. CT disappeared completely in 2 days in the enrichment from the Seal Beach site, without production of CF. It was concluded that enrichments from the 24-m depth at Savannah River and from Seal Beach had a high potential for CT degradation, and both enrichments were used to obtain isolates.

The enrichment culture from Seal Beach, Calif., was unable to degrade 1,1,1-trichloroethane, but it did degrade CT with glycerol as an alternative electron donor. Isolates from this material were capable of CT transformation, but were incapable of degrading CF. On aerobic minimal medium plates with acetate as the carbon source, isolated colonies spread out in a thin layer, with a yellow mound at the center, giving the appearance of fried eggs. On nutrient agar plates, they formed hard yellowish mounds that were difficult to extricate from the agar. Under a light microscope, all of the colonies appeared to be gram-negative rods about 0.4 by 1.7 μm in size.

A single isolate termed strain KC was selected from the Seal Beach enrichment for further characterization with commercial Minitek (BBL Microbiology Systems, Cockeysville, Md.) and Bacto (Difco Laboratories, Detroit, Mich.) test kits. The results provided in Table 1 indicate that this isolate belongs to the genus *Pseudomonas*. Culture purity was confirmed with a scanning electron microscope. Fatty acid analysis performed by Microbial ID, Inc., Newark, Del., confirmed the *Pseudomonas* classification and demonstrated a high correlation with the fatty acid profile for a library strain of *Pseudomonas stutzeri*. Microbial ID, Inc., reported a "similarity index" of 0.399 with *P. stutzeri*, 0.293 with *P. pseudoalcaligenes*, and 0.241 with *P. mendocina*. This index is a composite measure of the cumulative variance in the quantities and ratios of 10 long-chain fatty acids in comparison with a library standard. A correlation coefficient of 0.399 indicates a "high degree of correlation" (M. Sasser, Microbial ID, Inc.). Reported characteristics for *P. stutzeri* are also given in Table 1 for comparison.

Seven pure cultures were obtained and tested for CT transformation. The cultures evaluated were *P. stutzeri* ATCC 17832, *P. stutzeri* ATCC 39524, *P. aeruginosa* ATCC 10145, *P. fluorescens* A ATCC 17583, *P. fluorescens* B ATCC 17467, *P. mendocina* ATCC 17467, and *Hyphomicrobium* sp. strain GJ21 (courtesy D. Janssen, University of Groningen). All of these organisms grew well in medium D. Although some limited transformation of CT and CF formation was observed with *P. aeruginosa* ATCC 10145, none of these isolates was capable of significant transformation of CT in medium D. Thus, it appears that strain KC has unique characteristics for biotransformation of CT.

The importance of the electron donor and the electron acceptor for the transformation of CT was also evaluated. Very slow disappearance of CT was observed when oxygen or fumarate was provided as alternate electron acceptor for *Pseudomonas* strain KC. Transformation of CT was observed whether or not nitrate was present in excess of the amount required to completely oxidize the electron donor. Denitrification was confirmed by consumption of nitrate and acetate and production of protein (data not shown). The electron capture detector used to detect CT transformation also signaled simultaneous release and consumption of nitrous oxide, but no quantification was attempted. Transformation of CT did not appear to correlate with any particular phase of denitrification, but more work is needed in this area. Growth was a necessary but not sufficient condition for the transformation (see below). When grown cells were washed and suspended in a nongrowth medium, little or no transformation was observed.

Fate of radiolabeled CT. Three bottles containing medium D with 1.0 g of sodium acetate per liter and 0.67 g of sodium nitrate per liter were inoculated with colonies of strain KC grown on nutrient agar. Stock [^{14}C]CT in isooctane (10 μl) was added to these bottles and to two controls lacking strain KC. The distribution of ^{14}C after 2 days at 21°C is shown in Table 2. About half of the ^{14}C was recovered as carbon dioxide, and most of the balance was recovered in the nonvolatile acidified fraction. [^{14}C]CF recovered from the inoculated bottles was statistically indistinguishable from that of the controls.

Effects of medium composition on transformation. The possibility that strain KC might be useful in field applications was evaluated with groundwater from a shallow aquifer at Moffett Field, Calif. These studies (data not shown) suggested that (i) some characteristic of the Moffett water was inhibitory to the transformation of CT by strain KC, (ii) this inhibition could be partially alleviated by addition of phosphorus, and (iii) inhibition was aggravated by addition of trace metal solution TN2 to the groundwater. Figure 1 illustrates the sequence of hypotheses and experiments designed to understand the effects of solution chemistry on

TABLE 2. Distribution of radioactivity in denitrifying cultures of *Pseudomonas* sp. strain KC after a 2-day incubation with ^{14}C-labeled CT at 21°C

Test material	% of added radioactivity recovered as:					
	CT	CF	Nonvolatile fraction[a]	CO_2	Cell fraction	Total
Control[b]	97.0	1.3	0.9	0.8	0	100.0
Sample[c]	0.2 ± 0.2	1.3 ± 1.3	41.9 ± 5.8	46.9 ± 1.5	4.2 ± 0.6 bound 0.5 ± 0.1 sorbed	95.0

[a] Radioactivity remaining after sonication of an acidified sample for 1 h under a stream of nitrogen.
[b] Duplicates with an average total count of 10^6 dpm/bottle.
[c] Triplicates with a total count of $(1.1 \pm 0.1) \times 10^6$ dpm/bottle.

the transformation of CT. The results of these experiments are also summarized in Fig. 1.

To evaluate the medium constituents causing decreased transformation of CT, the effects of pH changes were first evaluated with medium D. The pH of one pair of bottles was left at 7.0 (by omitting the pH adjustment specified in the standard protocol), the pH of a second pair of bottles was adjusted to 7.5 with 3 N KOH, and the pH of a final pair was adjusted to 8.0, per the standard protocol. The results of these experiments are shown in Table 3. Transformation of CT by strain KC was inhibited at pH values of <8.0. When the pH was adjusted to 8.0, the medium became cloudy due to the formation of a white precipitate. Consequently, studies were performed to determine whether pH in itself or some component(s) of the precipitate prevented transformation of CT.

To assess the effects of the precipitate, medium D was prepared as usual, but the precipitate was removed by filtration with a prefilter after autoclaving and cooling. The pH of the filtered solution was then adjusted to 7.5 and 7.0 (with concentrated phosphoric acid), and the remaining medium was left at pH 8.0. Media at each pH were added to three bottles, which were then reautoclaved and cooled. To one bottle at each pH level, 0.15 ml of filter-sterilized solution TN 3 was added. This bottle and a second bottle at each pH level were inoculated with strain KC. The third bottle served as a control. Removal of precipitate alleviated the inhibition of CT degradation at all pH levels. However, inhibition occurred when trace nutrient solution TN3 was added to the filtered media, and this inhibition was most pronounced at pH 7.0. These effects are illustrated in Table 3.

To determine what component(s) of the precipitate and of

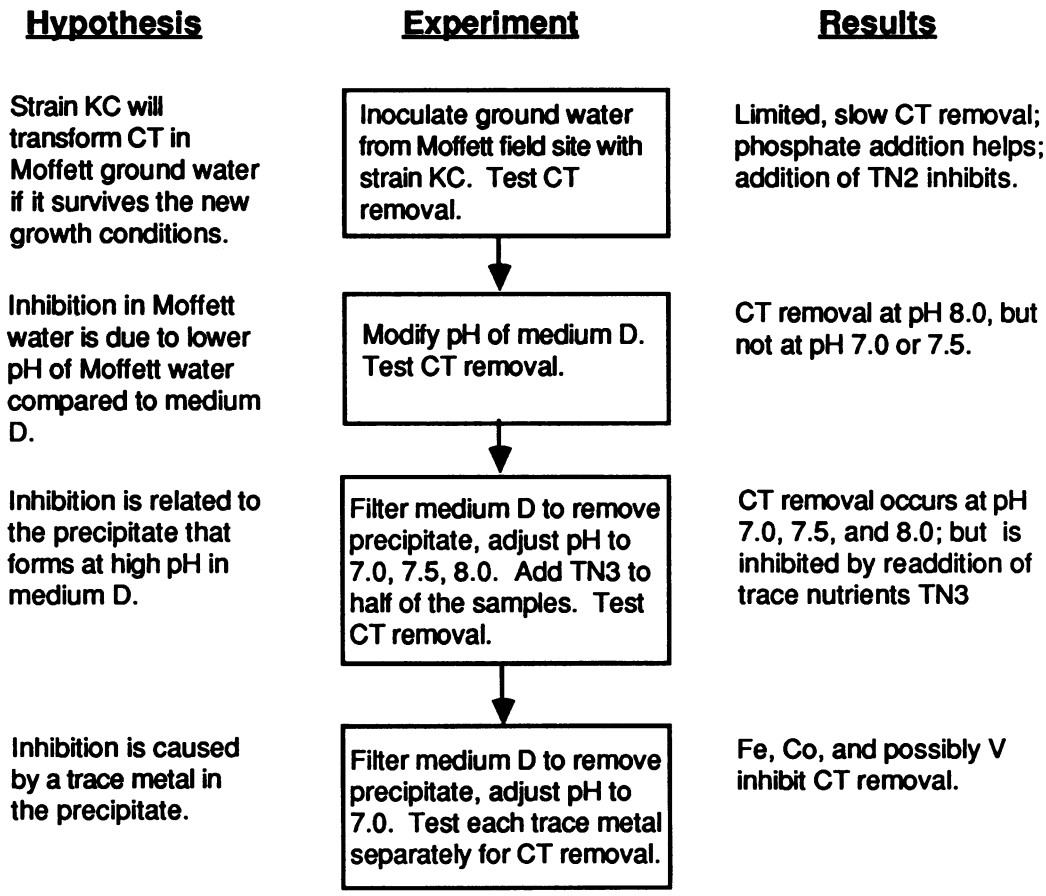

FIG. 1. Experimental procedure for evaluation of medium effects and the results obtained.

TABLE 3. Effect of trace metal solution TN3 on transformation of CT by strain KC when trace metals are added into solution following filtration of the media and pH adjustment

Day	Control (nmol of CT)	% of control CT					
		TN3 not added			TN3 added		
		pH 7	pH 7.5	pH 8	pH 7	pH 7.5	pH 8
0	209 ± 3	105	100	102	108	107	102
3	210 ± 3	36	14	0	107	34	27
7	197 ± 6	14	10	0	107	10	26

solution TN3 inhibited biotransformation of CT, trace metal stock solutions were prepared for each of the metals found in medium D. Medium D was prepared as described previously (0.5 g of sodium acetate per liter, 1.2 g of $NaNO_3$ per liter) and filtered through AW prefilters to remove the precipitate; the pH was then adjusted to 7.0, and the medium was dispensed into screw-cap bottles in the glove box. Different individual trace metals were then added, and the bottles were sealed, reautoclaved, cooled, spiked with CT, and inoculated with KC. The control was not inoculated with KC. CT was measured initially and after 5 days of incubation. The results of this experiment are shown in Table 4. Four trace metals had negative effects on the biotransformation of CT and/or on the growth of strain KC: Cu, Fe, V, and Co. No growth occurred when copper was present. Thus, increasing the pH of medium D to 8.0 in the enrichment cultures apparently resulted in the removal of toxic Cu. Adjustment of pH also removed other trace metals (Fe and Co) which apparently inhibited CT transformation by strain KC.

In a further experiment with ferrous iron, 2 liters of medium D (1.0 g of sodium acetate per liter, 0.67 g of $NaNO_3$ per liter) was prepared with 1 ml of trace nutrient solution TN2 per liter. The medium was adjusted to a pH of 8.0, autoclaved, cooled, filtered to remove precipitate, and neutralized to pH 7.0 with HCl. Media were then dispensed into 250-ml bottles which were reautoclaved and sealed; 1.0 ml of 0.01 M $FeSO_4 \cdot 7H_2O$ was added to some of the bottles, but not others, by filtration through a 0.2-μm filter (final added Fe concentration, 67 μM). All bottles, except for one control, were then inoculated with *Pseudomonas* sp. strain KC. The results are provided in Table 5. Both CT degradation and protein were monitored. As indicated, the presence of iron did not adversely affect protein production, and may have stimulated it, but ferrous iron again inhibited CT transformation.

TABLE 4. Effect of individual trace metals on biotransformation of CT by strain KC when individual trace metals are added separately back into filtered media after adjusting the pH to 7.0[a]

Trace metal tested	Initial CT in bottle (μg)	Final CT (μg)
Control	45	47
No addition	43	0
No addition	42	0
Cu	43	46[b]
Fe	41	42
V	44	15
Co	38	12
Mo	42	0
Ni	40	0
B	43	0
Mn	44	0
Zn	43	0

[a] Concentrations of trace metals added to filtered media: 1 μM Cu^{2+}, 2 μM Zn^{2+}, 1 μM Co^{2+}, 1 μM BO_3^{3-}, 0.4 μM Ni^{2+}, 1 μM MoO_4^{2-}, 6 μM Mn^{2+}, 5 μM Fe^{2+}, or 1 μM VO_3^-. Final CT measurements were taken after 5 days of incubation.

[b] No growth was observed in this bottle.

TABLE 5. Test of iron(II) additions on CT degradation and protein production

Day	Control CT (μg)	KC culture			
		No iron added		Iron added	
		CT (μg)	Protein (μg)[a]	CT (μg)	Protein (μg)[a]
0	26	30	0	22	0
2	25	27	13	18	15
4	24	2	19	18	25

[a] Average of duplicate samples.

DISCUSSION

A denitrifying *Pseudomonas* sp. capable of CT degradation was successfully isolated from Orange County aquifer materials. This is the first report of a CT-degrading denitrifier, and it demonstrates that at least one denitrifying organism can transform CT under the proper conditions. CT degradation is known to occur in denitrifying mixed cultures, but secondary nondenitrifying organisms are believed to be responsible for the observed transformations (5). On the whole, this work supports that view, inasmuch as six denitrifying *Pseudomonas* species obtained from the American Type Culture Collection and a denitrifying *Hyphomicrobium* sp., strain GJ21, were unable to carry out significant transformation of CT under the conditions tested.

The finding that *Pseudomonas* strain KC converted CT to carbon dioxide via a path that does not include CF (since CF was neither formed nor transformed) supports the view that CT biotransformation can proceed by at least two routes: one route leading to CF and dichloromethane production, and a second leading to carbon dioxide, as proposed by Egli et al. (5). However, *Pseudomonas* strain KC is apparently novel in its ability to direct CT toward carbon dioxide without simultaneous production of CF. This fact may have significance in the engineering of systems that direct CT to nonhazardous end products. A possible mechanism would be a rapid two-electron reduction of CT, leading to the production of a dichlorocarbene radical followed by spontaneous hydrolysis of the radical to give formate (C. Criddle and P. McCarty, submitted for publication). Formate could then be oxidized to carbon dioxide. Alternatively, the mechanism might involve the catalytic hydrolysis of the CT by an as yet unknown mechanism.

The inhibition of CT degradation by strain KC when reduced iron was provided in the growth medium indicates that the transformation of CT depends on the absence of soluble reduced iron (and possibly cobalt as well). The inhibitory effects of these trace metals on the transformation may explain the inability of strain KC to degrade CT in groundwater from the Moffett site.

One possible explanation for the observed inhibition is that induction of a trace metal scavenging mechanism is associated with the transformation of CT by *Pseudomonas* strain KC. The predicted equilibrium speciation of metals in medium D tends to support this hypothesis. The equilibrium

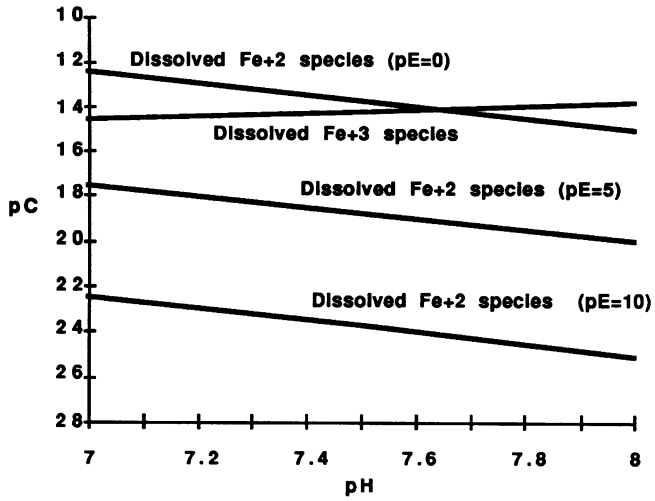

FIG. 2. Predicted solution speciation of iron in medium D at equilibrium as a function of pH and pE. Values were calculated with HYDRAQL (13).

speciation of medium D was modeled by using HYDRAQL, a program for the computation of chemical equilibria in aqueous batch systems (Fig. 2) (13). No thermodynamic information was available for vanadium species, but information for all of the other trace metal components was available in the thermodynamic data base of HYDRAQL. When the nitrate-nitrogen couple was allowed to control the electron activity of the system (by defining this couple in the data base), the predicted pE was 11.62 at pH 7. To evaluate a range of electron activities, however, the model was run at pEs of 0, 5, and 10, without defining either the nitrate-nitrogen couple or the acetate-carbon dioxide couple. These pE values represent a range of conditions that could exist under denitrification conditions. The model predicts that the two trace metals that will be least available in solution in medium D at pH 8.0 are copper (predicted $[Cu^{2+}]$, 1.7×10^{-13} M) and iron (predicted total dissolved iron concentration, $\sim 2 \times 10^{-15}$ to $\sim 3.0 \times 10^{-13}$ M, depending on pH and pE). Thus, the solution concentration of iron was apparently extremely low in these experiments. The model also predicts the following precipitated species at pH 8: $Fe_2(OH)_6$, $Ca_5(OH)(PO_4)_3$, $Mg_3(PO_4)_2$, $Zn_3(PO_4)_2$, $CoCO_3$, and $Ni(OH)_2$.

Some of the biochemical agents known to participate in the scavenging of iron are also reducing agents or participate in electron transfer reactions and could conceivably transform CT. Jansson (11) reported that nitrate-reducing bacteria (*P. fluorescens* and *Alcaligenes* sp.) increase the dissolution of iron and phosphorus sorbed to iron precipitates by excreting a high-molecular-weight compound. Most of the better-studied reductive systems for iron assimilation invoke a "taxi cab" mechanism, in which the microorganisms secrete a compound, such as a siderophore, that binds Fe^{3+} and returns it to the cell membrane where it is converted to Fe^{2+} by a reductase. The Fe^{2+} is then assimilated. The measured reduction potentials for hydroxamate siderophores are close to −400 mV, indicating that the reductase must be a strong reducing agent (6). There is some evidence that flavins are involved in this process and that the reductase lacks specificity (6). Flavins are reasonable candidates for the transformation since they are soluble and can carry out either one- or two-electron reductions (14). In summarizing their review of reductive metabolism of insecticides, Esaac and Matsumura (7) concluded that "most known reductive reactions for xenobiotics, namely dechlorination, sulfoxide, azo and nitro reductions, and N-demethylations are mediated by a soluble flavin *in-vitro*. Thus, it is logical to assume that such involvement of the soluble flavins could occur *in vivo* as well." Although it was not proven that flavins or other such agents were responsible for CT transformation by *Pseudomonas* strain KC, the experimental evidence is consistent with this hypothesis.

In addition to the pure culture obtained from Seal Beach aquifer material, several CT-degrading denitrifying enrichments were obtained from different aquifer materials, including an enrichment from a sample taken 24 m below the ground surface at the Savannah River site. Although no isolates were obtained from these enrichments, these findings suggest that capabilities for CT degradation must be widely dispersed in nature and probably result from the fortuitous reactivity of biochemical agents that normally serve other functions within the cell. The apparent dependence of CT transformation on the availability of iron in strain KC illustrates that useful fortuitous transformations may even occur in association with relatively obscure cell processes, such as mechanisms for the scavenging of trace nutrients. It also suggests that a wide range of capabilities are probably untapped and undiscovered, even in familiar organisms.

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