Localization and Characterization of the Carbon Tetrachloride Transformation Activity of *Pseudomonas* sp. Strain KC

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Previous research has established that *Pseudomonas* sp. strain KC rapidly transforms carbon tetrachloride (CT) to carbon dioxide (45 to 55%), a nonvolatile fraction (45 to 55%), and a cell-associated fraction (~5%) under denitrifying, iron-limited conditions. The present study provides additional characterization of the nonvolatile fraction, demonstrates that electron transfer plays a role in the transformation, and establishes the importance of both extracellular and intracellular factors. Experiments with ¹⁴C-labeled CT indicate that more than one nonvolatile product is produced during CT transformation by strain KC. One of these products, accounting for about 20% of the [¹⁴C]CT transformed, was identified as formate on the basis of its elution time from an ion-exchange column, its boiling point, and its conversion to ¹⁴CO₂ when incubated with formate dehydrogenase. Production of formate requires transfer of two electrons to the CT molecule. The role of electron transfer was also supported by experiments demonstrating that stationary-phase cells that do not transform CT can be stimulated to transform CT when supplemented with acetate (electron donor), nitrate (electron acceptor), or a protonophore (carbonyl cyanide *m*-chlorophenylhydrazone). The location of transformation activity was also evaluated. By themselves, washed cells did not transform CT to a significant degree. Occasionally, CT transformation was observed by cell-free culture supernatant, but this activity was not reliable. Rapid and reliable CT transformation was only obtained when washed whole cells were reconstituted with culture supernatant, indicating that both extracellular and intracellular factors are normally required for CT transformation. Fractionation of culture supernatant by ultrafiltration established that the extracellular factor or factors are small, with an apparent molecular mass of less than 500 Da. The extracellular factor or factors were stable after lyophilization to powder and were extractable with acetone. Addition of micromolar levels of iron inhibited CT transformation in whole cultures, but the level of iron needed to inhibit CT transformation was over 100-fold higher for washed cells reconstituted with a 10,000-Da supernatant filtrate. Thus, the inhibitory effects of iron are exacerbated by a supernatant factor or factors with a molecular mass greater than 10,000 Da.

Pseudomonas sp. strain KC is a natural aquifer isolate that rapidly transforms carbon tetrachloride (CT) to carbon dioxide and nonvolatile end product(s) without the production of chloroform under denitrifying conditions. The ability of *Pseudomonas* sp. strain KC to transform CT is dependent on iron-limiting conditions in the growth medium (1a, 7, 16). Addition of ferric iron to an actively transforming culture inhibits CT transformation (16). On the basis of these observations, we have previously hypothesized that an iron-scavenging system plays a key role in the transformation of CT by *Pseudomonas* sp. strain KC (1a, 16). Related species and denitrifying enrichments tested to date have been unable to transform CT when the cells are grown under the same conditions (1a, 7).

In this report, we evaluate the role of intracellular and extracellular components in CT transformation activity and describe partial purification of the extracellular factor(s). We characterize the extracellular factor(s) by size and iron sensitivity. We also identify formate as one of the nonvolatile CT transformation products, implicating electron transfer in the reaction mechanism. Additional evidence for the role of electron transfer is provided.

MATERIALS AND METHODS

Organisms. *Pseudomonas* sp. strain KC (DSM 7136, ATCC 55595), derived originally from aquifer solids from Seal Beach, Calif. (1a), is routinely maintained in our laboratories on nutrient agar plates. *Pseudomonas fluorescens* (ATCC 13525) was obtained from the culture collection of the Department of Microbiology at Michigan State University.

Chemicals, radioisotopes, and enzymes. Tetrachloromethane (CT; 99% purity) was obtained from Aldrich Chemical Co., Milwaukee, Wis. All chemicals used were American Chemical Society reagent grade (Aldrich or Sigma Chemical Co.). All water used in reagent preparation was of 18-M Ω resistance or greater. [¹⁴C]CT (3.4 mCi/mM) was provided by DuPont, NEN (Boston, Mass.). Chemical purity (99%) was determined by gas-liquid chromatography with a Poropak QS Durapak Porasil C column at 180°C and by gas chromatography with a 100/120-mesh column (10% Alltech CS-10 on Chromosorb W-AW) column at 60°C. Radiochemical purity was determined by gas chromatography with a Perkin-Elmer Megabore cyanomethylphenyl silicone column (PE N9312844) at 60°C, with 10 ml of N₂ carrier gas per min, and with trapping of the eluting gas. CT was detected with an electron capture detector, and radioactivity was determined by capturing gas fractions in Safety-Solve scintillation cocktail (RPI, Mt. Prospect, Ill.) and by liquid scintillation spectroscopy. Only one peak of radioactivity was detected, coeluting at 8 min with the CT peak. Purity was confirmed by reverse-phase high-performance liquid chromatography (HPLC; Gilson model 303 HPLC; Whatman C₁₈ Partisphere [5 μ m], 4.6 by 250 mm column, 0 to 100% acetonitrile gradient, flow rate of 1.5 ml/min). Fractions of eluant (1.5 ml) were collected directly into the scintillation cocktail. Purity (98.2%) was calculated as CT purity = [(cpm of CT peak)/(total cpm injected)] $\times$ 100. Antimycin A, carbonyl chloride *m*-chlorophenylhydrazone (CCCP), 2-heptyl-4-hydroxyquinoline (HQNO), formate dehydrogenase, and NAD were obtained from Sigma Chemical Company (St. Louis, Mo.).

Media. Medium D was prepared and dispensed in 28-ml serum tubes or modified 1-liter Wheaton bottles (1) as previously described (16). 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 of shaking (Lab-Line Orbital Shaker model 3590) under aerobic or denitrifying (N₂ headspace) con-

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ditions. For purification of supernatant components, strain KC was grown in simulated groundwater (SGW) medium (recipe provided by R. Skeen, Battelle Pacific Northwest Laboratory). SGW medium contained (per liter of deionized water) 0.455 g of $\text{Na}_2\text{SiO}_3 \cdot 9 \text{H}_2\text{O}$, 0.16 g of Na_2CO_3 , 0.006 g of Na_2SO_4 , 0.02 g of KOH, 0.118 g of $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 0.0081 g of $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 13.61 g of KH_2PO_4 , 1.6 g of NaOH, 1.6 g of NaNO_3 , 1.6 g of acetate, and 1 ml of trace element solution. The trace element solution contained (per liter of deionized water) 0.021 g of LiCl_2 , 0.08 g of $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 0.106 g of $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 0.6 g of H_3BO_3 , 0.123 g of $\text{Al}_2(\text{SO}_4)_3 \cdot 18 \text{H}_2\text{O}$, 0.11 g of $\text{NiCl}_2 \cdot 6 \text{H}_2\text{O}$, 0.109 g of $\text{CoSO}_4 \cdot 7\text{H}_2\text{O}$, 0.06 g of TiCl_4 , 0.03 g of KBr, 0.03 g of KI, 0.629 g of $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, 0.036 g of $\text{SnCl}_2 \cdot 2\text{H}_2\text{O}$, and 0.3 g of $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$. The pH of SGW medium was adjusted to 8.2 with 3 N KOH. The resulting medium was autoclaved at 121°C for 20 min and then transferred to a Coy anaerobic glove box for degassing.

Analytical methods. CT was assayed by removal of the samples of headspace gas above liquid samples and injection of the samples onto a gas chromatograph as described previously (16). External calibration standards were prepared by addition of a primary CT standard in methanol to generate a five-point calibration curve bracketing the concentrations in the assay samples (16). Formate and other ions were assayed by ion chromatography (Dionex model 2000i/SP ion chromatograph with suppressed conductivity detection equipped with a Sarsap AN 1 column and utilizing a 1.8 mM bicarbonate–1.7 mM carbonate mobile phase at 1 ml/min). Chromatograms were recorded, and data were integrated with a Spectra Physics model SP 4270 integrator. External standard calibration curves were prepared by diluting primary ion standards into secondary water standards having the same ionic composition as the test samples. Measurements of pH were made with an Orion model 720A pH meter. Protein was assayed by the modified Lowry method, with bovine serum albumin as the standard (8).

Generation of CT transformation products. A 1-liter culture of *Pseudomonas* sp. strain KC (24 h of growth, 316 µg of protein per ml) was spiked with 64 µg of ^{14}C -labeled CT and transformation of the CT was monitored to completion (approximately 24 h). Cells were removed by centrifugation and filtration of supernatant through a 0.45-µm-pore-diameter filter. The cell supernatant was lyophilized to dryness, resuspended in 30 ml of distilled water, and filtered through a 30,000-Da and then 3,000-Da molecular mass cutoff filtration device (Centriprep 30 and Centriprep 3, respectively; Amicon). The 3,000-Da (molecular mass) filtrate was lyophilized to dryness and used to determine the identity of the nonvolatile product(s).

Ion-exchange chromatography. Anion-exchange chromatography was performed with the nonvolatile transformation products. Samples of dry nonvolatile product (1.0 g) were resuspended in 1 ml of 2 mM potassium phosphate buffer. Samples (450 µl at pH 5.0 or 8.0) were then loaded on a Pharmacia (Uppsala, Sweden) DEAE-Sephadex A-25 anion-exchange column (16 by 1 cm; bed volume, 9 ml) that had been previously equilibrated with 2 mM potassium phosphate buffer at pH 5.0 or 8.0. A 0 to 250 mM NaCl gradient was generated with a Bethesda Research Laboratories (Gaithersburg, Md.) model GA-0979 gradient former (total volume, 83 ml). The column was run under natural gravity flow, and 4-ml fractions were collected and assayed for radioactivity. An identical column and gradient were run to determine the elution profile of formate. Radioactivity was determined by counting 1-ml samples in 20 ml of Safety-Solve scintillation fluid (RPI) on a Packard Tri-Carb liquid scintillation spectrometer (Downers Grove, Ill.). Nonradioactive ion standards were monitored by ion chromatography as described previously.

Fractional distillation of ^{14}C -labeled transformation products. CT transformation products were subjected to fractional distillation under vacuum. A sample of lyophilized Centriprep purified nonvolatile product(s) (500 mg [dry weight], 155,000 cpm) was resuspended in 7.0 ml of distilled water and acidified from pH 8.56 to 2.71 by addition of 400 µl of concentrated phosphoric acid. Carbon dioxide was removed by flushing with nitrogen at room temperature for 60 min. Fifty-four percent of the ^{14}C counts were removed from the aqueous phase by this procedure. The remaining aqueous sample (7.0 ml, 11,285 cpm/ml) was transferred to a 60-ml Bantam-ware microdistillation device equipped with Teflon valves and a Teflon thermometer fitting. The apparatus was connected to a vacuum trap placed in a dry ice and methanol bath and connected to a vacuum pump. The sample was slowly heated with a rheostat-controlled heating mantle to 99.5°C, the boiling point of water under atmospheric pressure in our laboratory. Vacuum was initiated and transfer was allowed to proceed for 30 min, after which samples of the distillate (volume, 1.25 ml; 180 cpm/ml) and the remaining liquid in the boiler (volume, 5.56 ml; 14,150 cpm/ml) were assayed for radioactivity. The apparatus was reconnected and heated until the temperature increased to 100.6°C, the boiling point of protonated formate. Vacuum was initiated and maintained for 15 min, during which time a total of 2.52 ml of additional distillate was collected. Distillate was only collected for the 15-min period, because after 15 min, the temperature of the mixture began to exceed 100.6°C. The distillate contained 3,125 cpm, or 2.1% of the total radioactivity of the lyophilized material.

Enzymatic production of carbon dioxide. To determine the identity of the distilled nonvolatile material, formate dehydrogenase was used to convert putative ^{14}C -formate to $^{14}\text{CO}_2$ (10). To 2.5 ml of sample, the following additions were made in order: 320 µl of 400 mM potassium phosphate buffer (pH 7.0), 250 µl of 7-mg/ml NAD (Sigma Chemical Co.), and 500 µl of 1,923-mU/ml formate dehydrogenase (Sigma Chemical Co.). The reaction proceeded for 1 h at 20°C

with 150-rpm shaking. The carbon dioxide level was determined by a modification of the procedure of Zehnder and Brock (19). After incubation, the sample was acidified to pH 2 by addition of 100 µl of 6 M HCl in a sealed reaction vessel. The vessel was connected with Tygon tubing to a vessel containing 1.3 ml of 1 N KOH. The transfer tubing was placed into the aqueous base, and the sample was flushed with 15 ml of nitrogen per min for 25 min. Sixty-two percent of the distilled nonvolatile material was recovered as $^{14}\text{CO}_2$ by this procedure.

Bioassay for the secreted factor(s) with *P. fluorescens*. Tatara et al. (17) discovered that rapid CT 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 (10,000 rpm, 10 min, Beckman SS-34 rotor, 4°C) and resuspended to 1/10-th the original culture volume in medium D to a cell density of approximately 2×10^9 CFU/ml. Five hundred microliters of the resulting $10 \times$ 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 of the anaerobic glove box (the interlock autocycle was run three times). Samples were sealed under N_2 headspace in 28-ml Balch tubes and spiked with CT. Levels of CT were monitored by headspace gas chromatography as previously described (16).

Fractionation of CT transformation activity. To localize the CT transformation activity of strain KC, actively transforming cultures were fractionated by centrifugation and ultrafiltration. Cultures grown for 24 h in SGW medium under denitrifying conditions were first screened for CT transformation activity. Typically, about 300 ml of actively transforming culture (protein, 15.3 ± 0.2 µg/ml [mean $\pm$ standard deviation]) was dispensed into degassed Oak Ridge-style centrifuge tubes in the anaerobic glove box. This type of centrifuge tube excludes oxygen and can maintain the strictly anaerobic conditions required for methanogenesis during 10-min centrifuge runs (3). Cells were harvested by centrifugation (10 min at 10,000 rpm with a Beckman SS-34 rotor at 4°C), and the supernatant was filtered through a 0.2-µm-pore-diameter filter. Occasionally, the cell supernatant was capable of CT transformation. In these instances, the supernatant was further fractionated by filtration through Amicon 10,000- and 500-Da molecular mass cutoff filters with an Amicon model 8400 stirred pressure cell (500-ml volume, N_2 pressure of 45 lb/in², room temperature) in the anaerobic glove box (95% N_2 –5% H_2 atmosphere). The filtrate and retentate of each filter were assayed for CT transformation activity. The CT transformation assay was performed with 4.5-ml samples of the fractions with and without reconstitution with *Pseudomonas* sp. strain KC cells or *P. fluorescens* cells. Samples were dispensed under N_2 and spiked with CT to 10 µg/liter. CT levels were assayed by gas chromatography as described above.

In general, cell supernatant and its filtered fractions (10,000- and 500-Da filtrate) did not mediate appreciable CT transformation alone. Rapid transformation was usually obtained only when these fractions were combined with viable whole cells. For this cell-requiring activity, filtrate and retentate from the 10,000- and 500-Da filters were combined with cells of strain KC before being assayed for CT transformation or they were tested in the *P. fluorescens* bioassay.

Acetone purification of transformation activity. Filtrate passed through an Amicon 500-Da filter and was lyophilized to dryness (yield of 12 mg [dry weight] of filtrate per ml). The lyophilized filtrate was suspended in approximately 5% of the original filtrate volume (254 mg of lyophilized filtrate per ml) in ultrapure deionized water. The sample was then transferred to a 28-ml test tube, and 9 ml of HPLC-grade acetone per ml was added to the sample. Samples were stored at 4°C for 2 h 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 polytetrafluoroethylene filter (Gelman Acrodisc) to remove any particulate matter. The acetone water phase was then evaporated to dryness under nitrogen at room temperature. Approximately 80% of the bioassay activity was recovered in the dried acetone fraction. The yield was approximately 7 µg of partially purified material per mg of dry 500-Da filtrate precipitated.

Effects of iron. To assess the effects of iron on CT transformation, an actively transforming culture was fractionated as described above, individual fractions were spiked with 0 to 100 µM iron (as ferric ammonium sulfate) plus 10 µg of CT per liter, and each fraction was assayed for CT transformation. Both the cell-free transformation activity and the cell-requiring transformation activity were evaluated for their sensitivity to iron after fractionation. For the cell-requiring activity, 10,000-Da filtrate and 500-Da filtrate were tested for activity by being recombined (reconstitution) with cells of strain KC as described above.

Determination of iron-binding activities. To determine the spectrum of iron-binding activities produced by strain KC, cultures (24-h growth, medium D, 141 µg of protein per ml) were fractionated by cell removal and ultrafiltration as described above. The culture supernatant, 10,000-Da filtrate, 500-Da retentate, and 500-Da filtrate were assayed for total iron-binding activity by the method of Schwyn and Neilands (13), the Csáky assay (2) for hydroxamate-class siderophores, and the Rioux assay (12) for catechol-class siderophores.

Effects of electron donor and electron acceptor levels on CT transformation. A dependence of CT transformation on the growth phase has been observed when *Pseudomonas* sp. strain KC is grown in medium D with excess acetate (16). CT transformation rates decreased as cultures entered the stationary growth phase. To determine if addition of an electron donor (acetate) or electron acceptor (nitrate) would stimulate CT transformation in stationary-phase cul-

FIG. 1. Elution of ^{14}C -labeled nonvolatile CT transformation products from a DEAE-Sephadex A-25 column. Forty-five percent of the radioactivity added to the culture was recovered in the nonvolatile (non vol) fraction. Over 95% of the nonvolatile radioactive material applied to the column was recovered in the effluent fractions. The formate standard elution is shown for comparison.

tures of strain KC, cultures were grown in medium D to the stationary phase (72 h, 152 μg of protein per ml). The culture was subdivided into 10-ml aliquots and dispensed into 28-ml Balch tubes under N_2 . Cultures were spiked with CT and shaken at 150 rpm. Levels of CT were determined by gas chromatography as described previously. After 10 min, subsets of the samples received 2 mM acetate or nitrate. One set of cultures received no additions and served as an unstimulated control.

Effects of protonophores and electron transfer inhibitors. The effects of the protonophore CCCP on CT transformation were assessed in growing (48-h culture, 123 μg of protein per ml) and stationary-phase (72-h culture, 153 μg of protein per ml) cultures of strain KC. In addition, the effects of antimycin A and HQNO were assessed with growing (48-h) cultures of strain KC. Cultures were grown in medium D to the appropriate growth phase, and inhibitors were added 10 min prior to spiking cultures with CT. CT levels were monitored by gas chromatography as described previously.

RESULTS

Chromatographic analysis of the nonvolatile CT transformation products. Transformation of CT by *Pseudomonas* sp. strain KC generates CO_2 , a cell-associated product(s), and a nonvolatile product(s) (1a, 7). We sought to determine the identity of the nonvolatile transformation product(s) by purifying and characterizing ^{14}C -labeled transformation products. Anion-exchange chromatography was able to resolve two ^{14}C -labeled CT transformation products in the nonvolatile fraction at pH 8. As shown in Fig. 1, one peak eluted in the void volume from a DEAE-Sephadex column, and the other peak coeluted with a formate standard. At pH 5.0, only a void volume peak was observed. Cation-exchange chromatography (Pharmacia CM-Sephadex C-50) was performed on the nonvolatile products, and a single void volume peak was observed. This suggested that one of the nonvolatile products was negatively charged at alkaline pH and was protonated at pH 5.0.

Fractional distillation and enzymatic conversion of CT transformation products. The results of the chromatographic analysis of the nonvolatile CT transformation products were consistent with the view that one of the CT transformation products was an organic acid, and formate was a likely candidate. Fractional distillation of protonated transformation products showed that approximately 54% of the transformation products was carbon dioxide (purged under acidic conditions by flushing with nitrogen). The fractional distillation also revealed the presence of a transformation product which boiled at 100.6°C . (A total of 3.9% of the nonvolatile counts

FIG. 2. CT transformation by a mixture of *Pseudomonas* sp. strain KC cells and 500-Da (MW) filtrate. Cells were grown for 24 h in medium D (76 μg of protein per ml).

[non- CO_2 counts] was recovered in the distillate in 15 min.) The boiling point of protonated formate is 100.67°C (18). To confirm formate production, the distillate was recovered and subjected to enzymatic conversion with formate dehydrogenase. Formate dehydrogenase specifically converts formate to CO_2 in the presence of NAD (10). After incubation with formate dehydrogenase, 62% of the radioactivity in the 100.6°C distillate was recovered as $^{14}\text{CO}_2$. These data indicate that formate is one of the nonvolatile CT transformation products produced by strain KC.

Reconstitution of fractionated CT transformation activity.

Figure 2 illustrates CT transformation by washed cells of strain KC, 500-Da 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 the filtrate fraction, indicating that the secreted factor(s) required for transformation is small, with a nominal molecular mass of less than 500 Da. It should also be noted, however, that, in some cases (see, for example, Fig. 2), CT transformation activity with the partially purified cell-free supernatant was observed. Tatara et al. (17) reported a pseudo-first-order rate constant of $0.03 \pm 0.03 \text{ min}^{-1}$ 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 CT degradation that can be achieved by the cell free activity is shown in Table 1; however, it must be stressed this example is not an average but rather is

TABLE 1. Transformation kinetics for fractionated cell-free activity

Fraction	Pseudo-2nd-order rate coefficient (liters/mg of protein [dry wt]/day) ^a
Example 1	
Complete culture.....	0.54
Supernatant.....	6.1
10,000-Da filtrate.....	21.3
Example 2	
Complete culture.....	1.3
500-Da filtrate.....	2.1

^a Determined by the method of Tatara et al. (16).

a characterization of the activity on two of the relatively rare occasions in which significant cell-free activity was observed. Additional research is needed to understand the conditions required for the cell-free activity.

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 with *P. fluorescens* to track production of the secreted activity (17). *P. fluorescens* cells were preferred for the bioassay because, unlike strain KC, they are incapable of generating additional secreted transformation activity during the assay.

Purification of secreted factor(s) for the cell-requiring CT transformation activity. The <500-Da component(s) of the cell-requiring system was stable for up to 7 days in SGW medium (17) and retained its activity as a lyophilized dry material stored desiccated at -20°C for over 20 days. Anion-exchange perfusion chromatography revealed the presence of at least five constituents in the 500-Da 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 CT transformation activity. This salt concentration was in the center of the range in the salt gradient in which elution of the major protein peaks occurred. Ammonium sulfate precipitation of the activity was also unsuccessful, possibly because of 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 (vol/vol). 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. Note that, with this technique, all samples were assayed by reconstitution with *P. fluorescens* cells (2×10^8 CFU/ml) in SGW medium. The following amounts of CT (mean ± 1 standard deviation) were degraded by the acetone extraction fractions *P. fluorescens* fraction with no additions, 0.001 ± 0.001 μg ; positive control (10 mg of lyophilized 500-Da filtrate), 0.047 ± 0.006 μg ; acetone supernatant, 0.049 ± 0.008 μg ; and acetone pellet, 0.013 ± 0.004 μg .

Effects of iron and fractionation of iron-binding activities. CT transformation by strain KC is inhibited by iron, and the possible role of a siderophore or siderophorelike agent has been previously proposed (1a, 16). We sought to characterize the fractions generated during purification in terms of siderophore levels and sensitivity to iron inhibition. To track siderophore activity in the various size fractions, we used the universal siderophore assay of Neilands (13), the Csáky assay for hydroxamate-class siderophores (2), and the Rioux assay for catechol-class siderophores (12). The results are shown in Table 2. The 500-Da 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-Da molecular mass filter showed a concentration of iron-binding activity of all three classes, indicating the production of multiple iron-binding activities by strain KC.

In marked contrast to the complete culture of *Pseudomonas* sp. strain KC, 10,000-Da filtrate and 500-Da filtrate did not exhibit high levels of iron sensitivity. Whole-culture activity is inhibited by iron levels as low as 1 μM , but the combination of 10,000-Da filtrate and washed cells was not inhibited by 100-fold-higher iron levels. This indicates that a high-molecular-

TABLE 2. Fractionation of iron-binding activities produced by *Pseudomonas* sp. strain KC^a

Fraction	Method of measuring iron-binding activity		
	Rioux (μM 2,3-DHBA) ^b	Csáky (ΔA_{520})	Neilands (ΔA_{630})
Blank	0	0	0
Supernatant	3.08	0.04	0.38
10-kDa filtrate	2.18	0.02	0.39
0.5- to 10-kDa retentate	3.27	0.59	0.41
<0.5-kDa filtrate	1.57	0.04	0.34

^a Measured after 24 h of growth in medium D (141 μg of protein per ml). For references, see text.

^b 2,3-DHBA, 2,3-dihydroxybenzoic acid.

mass secreted factor(s) (>10,000 Da) exacerbates or is responsible for the inhibitory effects of iron.

Effects of acetate and nitrate addition on CT transformation. Transformation of CT by strain KC is dependent upon the culture growth phase (16). Maximal CT transformation occurs during the logarithmic growth phase and declines after 48 h (16). To determine the role of electron donors and electron acceptors, acetate and nitrate were added to late-stationary-phase cells. Late-stationary-growth-phase cells showed little CT transformation, but addition of either acetate or nitrate stimulated transformation activity. It is possible that the acetate is used for respiration, perhaps in combination with residual organic electron acceptors in the culture broth. Similarly, addition of nitrate may have facilitated respiration by utilizing organic electron donors. The cells in this experiment were grown under equimolar concentrations of electron donor and electron acceptor.

Effect of inhibitors on CT transformation. To further assess the role of respiration in CT transformation, the effects of the respiratory chain inhibitors antimycin and HQNO and the protonophore CCCP were examined. HQNO inhibits respiration by blocking electron transport between coenzyme Q and cytochrome *b*, and antimycin inhibits electron transport between cytochrome *b* and cytochrome *c* or cytochrome *o* (5, 6, 14). No inhibition of CT transformation was found in the presence of antimycin, HQNO, or CCCP. This indicates that coupling via a proton gradient does not directly drive CT transformation; consequently, if coupling to respiration occurs, the linkage to the electron transport chain is above the level of cytochrome *b*.

To determine whether electron flux through the respiratory chain enables CT transformation, stationary-phase nontransforming cells were treated with the protonophore CCCP. As shown in Fig. 3, addition of CCCP stimulated rapid CT transformation. CCCP uncouples respiration from ATP synthesis, and with the extrusion of protons no longer poised against the phosphorylation potential, rapid electron transfer down the respiratory chain can occur (4, 9, 11, 15).

DISCUSSION

The results presented above indicate that CT transformation proceeds via a complex mechanism that normally involves both cellular and secreted components. A plausible model would involve (i) production and export of a CT-transforming factor(s) from the cell in response to iron limitation, (ii) deactivation of the factor(s) upon transformation of CT, and (iii) reactivation of the factor(s) at the cell membrane. The rare cell-free activity may involve additional or modified secreted components, possibly metal-containing cofactors, which substi-

FIG. 3. Uncoupler-induced CT transformation by resting cells of *Pseudomonas* sp. strain KC. CCCP was added 10 min before CT was added. At the same time, the stationary-phase cell control received a mock spike of ethanol only. Cells were grown for 72 h in medium D (152 μ g of protein per ml).

tute for the cellular components. Alternatively, the active agent of transformation itself may be modified under some conditions.

We had previously hypothesized that the secreted factor(s) might be a siderophore because of its induction under iron-limiting conditions (1a, 16). The present study suggests a more complex picture. Given that copper is required for CT transformation by strain KC (16), it is possible that the secreted activity may involve a metal-containing cofactor. The metal-containing cofactors vitamin B₁₂, coenzyme F₄₃₀, and heme have been shown to catalyze reductive dechlorination of chlorinated compounds when coupled to reductants such as titanium(III) citrate (4). It is also possible that such cofactors play a regenerative role for secreted activity.

Strain KC generates a wide range of secreted iron-binding activities. The fact that iron inhibition of CT transformation was alleviated when the supernatant was filtered through a 10,000-Da filter suggests that sensitivity to iron inhibition is conferred by a high-molecular-mass factor(s). This factor(s) may participate in iron-scavenging activities. The insensitivity of the 10,000-Da filtrate to iron inhibition suggests that the small secreted CT-transforming factor(s) either does not play a direct role in iron binding or is still able to transform CT after binding iron. A possible explanation for these observations is that the normal physiological role of the low-molecular-mass CT-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 CT. Additional experimental evidence is needed to confirm or refute this hypothesis.

Of special interest is the transformation of CT to formate and carbon dioxide. Production of formate from CT requires a two-electron ($2e^-$) transfer: $CCl_4 + 2e^- + 2H_2O = HCOOH + 2HCl + 2Cl^-$. In contrast, production of carbon dioxide from CT is a net hydrolysis requiring no net electron transfer: $CCl_4 + 2H_2O = CO_2 + 4HCl$. It seems possible that CO₂ could be produced by the oxidation of the formate, as proposed by Criddle et al. (1a). However, ¹⁴C₂O₂ is recovered even when [¹⁴C]CT transformation is conducted in the presence of

a large pool of unlabeled formate, indicating that formate is not an intermediate in the pathway to CO₂ (2a). Thus, the mechanism of carbon dioxide production remains enigmatic. Nevertheless, the present findings do indicate that the reaction is dependent upon some kind of energy input. Stimulation of resting cells by the addition of electron donor, electron acceptor, and a protonophore suggests that the role of cells is to deliver reducing power to the secreted factor(s) that ultimately transforms CT. Additional research is needed to elucidate the structure of the secreted factor(s) and to clarify the mechanism of regeneration.

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