

Conversion of Kepone by *Methanosarcina thermophila*

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Abstract

Acetate-grown cultures of *Methanosarcina thermophila* converted uniformly labeled [¹⁴C]Kepone to polar and nonpolar products with 86% of the Kepone degraded within the first 10 days. The titanium(III) citrate-reduced CO dehydrogenase enzyme complex isolated from *M. thermophila* also catalyzed the conversion of Kepone to polar and nonpolar products with a similar pattern as seen with whole cell cultures. Similar patterns of soluble Kepone decomposition products were obtained with reduced vitamin B₁₂, reduced corrinoid cofactor (factor III) isolated from the CO dehydrogenase enzyme complex, and reduced coenzyme F₄₃₀ isolated from the methyl coenzyme M methylreductase of *M. thermophila*.

Keywords: Kepone; *Methanosarcina thermophila*; Bioconversion

1. Introduction

Microbial reductive dehalogenation occurs in anaerobic environments where highly chlorinated hydrocarbons are generally more easily dechlorinated anaerobically, many under methanogenic conditions. Pure culture studies have focused on the reductive dehalogenation of simple (C₁ and C₂) compounds [1,2].

Kepone (decachlorooctahydro-1,3,4-metheno-2H-cyclobuta(cd)pentalen-2-one) (Fig. 1) is a highly chlorinated, extremely toxic insecticide [3]. Waste-

water discharges during the manufacture severely contaminated the James River [3] where Kepone persists in anaerobic sediments and continues to be a major health hazard. Here we show that acetate-metabolizing cultures of *Methanosarcina thermophila* catalyze the conversion of Kepone to polar and nonpolar products.

2. Materials and methods

2.1. Organism and culture conditions

Cultures of *M. thermophila* [4] (100 ml) were grown on acetate as described [5]. A stock solution of [¹⁴C]Kepone (6.1 mCi/mmol; 99.9% radiochemical purity) (Sigma Chemical Co., St. Louis, MO) was prepared in 50% anaerobic ethylene glycol (final

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concentration 7.9 mg Kepone/ml (w/v)). [^{14}C]-Kepone was added to the growth medium to a final concentration of 0.79 mg/ml. At specified intervals during growth, 1.0 ml of culture supernatant was withdrawn and assayed as described below. Growth was assessed by following methane production.

2.2. Enzyme and cofactor assays

The standard assay was performed at 50°C in 8 ml amber vials stoppered with butyl rubber stoppers and pressurized to 101 kPa with N_2 [6]. The standard reaction mixture (0.2 ml) contained (final concentrations): 50 mM TES (*N*-tris(hydroxymethyl) methyl-2-aminomethanesulfonate) (pH 6.8) which contained 10% (v/v) ethylene glycol, 70 μM [^{14}C]Kepone, 5 μM of either factor III, vitamin B_{12} , or coenzyme F_{430} , and 10 mM titanium(III) citrate. Titanium(III) citrate was prepared as described previously [7]. The reaction mixture was incubated at 22°C for 15 min before initiation of the reaction by addition of [^{14}C]Kepone and transfer to a 50°C water bath.

2.3. Analytical techniques

To quantitate total soluble radioactivity in the whole cell culture studies, 0.1 ml of culture supernatant was assayed by liquid scintillation counting using ScintiVerse fluid (Fisher Scientific, Pittsburgh, PA). Detection of soluble radioactive compounds was performed by spotting 0.5 ml of culture supernatant onto glass-backed silica gel GF thin-layer chromatography (TLC) plates (20 × 20 cm) (Fisher) which were then developed with hexanes:acetone (3:1). The radioactive spots were detected by autoradiography. Radioactive areas were scraped from the TLC plate and quantitated by liquid scintillation counting.

Total volatile radioactive products were quantified using glass scintillation vials fitted with butyl rubber stoppers as described [8]. The gas phase (1.0 ml) from the culture was added to 0.5 ml of scintillant through the stopper. To identify the volatile products, gas chromatography using a thermal conductivity detector equipped with a Poropak Q column [9] was used to separate gaseous products before collection in scintillation fluid.

2.4. Materials

Coenzyme F_{430} was extracted from purified methyl coenzyme M methylreductase of *M. thermophila* as described previously [10]. Factor III was a kind gift of Dr. Erhard Stupperich. Vitamin B_{12} was purchased from Sigma. *M. thermophila* CODH enzyme complex was purified as described previously [11].

3. Results and discussion

Acetate-metabolizing cultures of *M. thermophila* converted uniformly labeled [^{14}C]Kepone to at least three non-volatile products resolved by TLC (Fig. 2). Approximately 85% of the Kepone was converted within the first 10 days without an appreciable lag (Fig. 3A). The major conversion product of Kepone was a polar compound which remained at the origin of the chromatogram. The polar products appeared at a rate inversely proportional to Kepone disappearance followed by a slower rate for conversion of the polar products to a compound or compounds undetectable by TLC. A polar Kepone decomposition product migrated to just above the origin (Fig. 2); however, very little radioactivity was detected in this product throughout the experiment (data not shown). Low but significant radioactivity was present in the non-polar product that migrated near the solvent front (Fig. 2). This product appeared without a lag and the concentration did not change significantly over 90 days (Fig. 3A).

A radioactive gas was produced in the cultures that paralleled total gas formation (Fig. 3B) and was identified as methane (Fig. 4). The production of other volatile compounds not detected by this gas

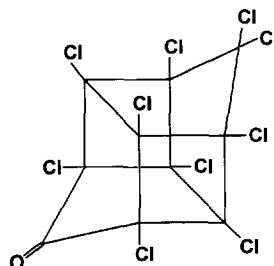


Fig. 1. Structure of Kepone (1,1a,3,3a,4,5,5,5a,5b,6-decachlorooctahydro-1,3,4-metheno-2H-cyclobuta[cd]pentalen-2-one).

chromatography procedure cannot be ruled out. No significant radioactivity was detected in methane until day 9 although most of the Kepone was converted. This result suggests that radioactive methane was derived from an intermediate product of Kepone decomposition, possibly the polar compound or compounds.

After 90 days, less than 1% of the radioactivity originally present in Kepone was accounted for in methane and about 59% and 4% in the polar and non-polar products respectively. Undegraded Kepone accounted for 11%. Thus, 74% of the radioactivity originally present in Kepone was recovered after 90 days incubation. The unaccounted 26% of radioactivity could result from inherent errors in quantitation or the inability to detect yet other decomposition products with the methods used. Kepone was completely stable in uninoculated culture medium (Fig. 2). The results suggest that Kepone is converted primarily to polar and nonpolar products in cultures of *M. thermophila* growing on acetate as the energy source.

The CO dehydrogenase complex from acetate-grown *M. thermophila* contains two enzyme components [12]. In addition to iron-sulfur centers, one component contains nickel (Ni/Fe-S component) and

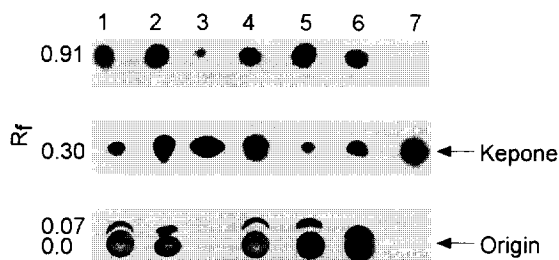


Fig. 2. Composite thin-layer autoradiogram of products obtained from the conversion of uniformly labeled [^{14}C]Kepone. Samples (10 μl) were spotted onto TLC plates and processed as described in Section 2. Lane 1: sampled from the supernatant of a 90-day acetate-grown culture of *Methanosarcina thermophila* which contained labeled Kepone (0.79 mg/ml). Lane 2: sampled from a standard reaction mixture (see Section 2) which also contained Ti(III) citrate (10 mM), labeled Kepone (70 μM), and 0.5 mg of purified CO dehydrogenase enzyme complex. Incubation was for 2 h at 50°C. Lane 3: same as lane 2 except Ti(III) citrate was omitted and CO replaced N_2 . Lanes 4–6: same as lane 2 except 5 μM of either factor III (lane 4), vitamin B_{12} (lane 5), or coenzyme F_{430} (lane 6) replaced the enzyme. Lane 7: same as lane 2 except the enzyme was omitted. R_f values are indicated.

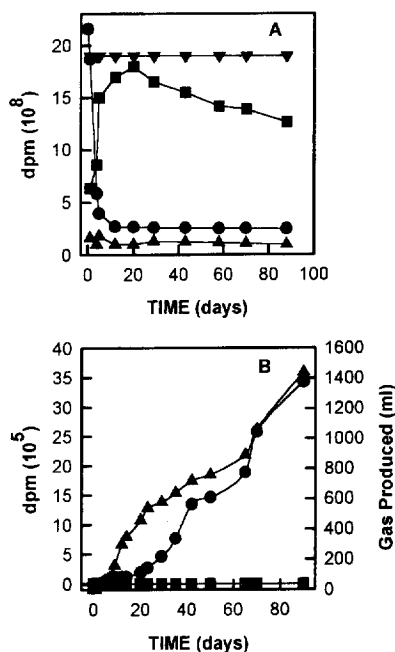


Fig. 3. Time course for the conversion of uniformly labeled [^{14}C]Kepone to soluble and volatile products by an acetate-grown culture of *Methanosarcina thermophila*. (A) Acetate (100 mM final concentration) and labeled Kepone (0.79 mg/ml) were added to the culture at the start of the experiment. The culture was supplemented with acetate on days 7, 15, 34, and 62. At the indicated times, 0.5 ml of culture supernatant was chromatographed as described in Fig. 2. Radioactivity in the spots containing Kepone (\bullet), and the polar (\blacksquare) and nonpolar (\blacktriangle) products (see Fig. 2 and text) were quantitated as described in Section 2. Values are the cumulative radioactivity from the 100 ml culture. The control (\blacktriangledown) was medium containing labeled Kepone (0.79 mg/ml) without cells. (B) At the indicated times, 1.0 ml of the gas phase from the culture described in A was withdrawn and the radioactivity quantitated as described in Section 2. The values shown are cumulative gas produced (\blacktriangle) and cumulative radioactivity in the gas phase (\bullet) from the 100 ml culture. The control (\blacksquare) was medium containing labeled Kepone (0.79 mg/ml) without cells.

the other (Co/Fe-S component) contains a vitamin B_{12} analog called factor III (Co α -[α -(5-hydroxybenzimidazolyl)]-Co-cyanocobamide).

The titanium(III) citrate-reduced CO dehydrogenase complex catalyzed the conversion of Kepone to at least three products with the same R_f values as the products observed in acetate-metabolizing cultures of *M. thermophila* (Fig. 2). No conversion occurred if the enzyme complex was omitted from the reaction mixture (data not shown). The results suggest that

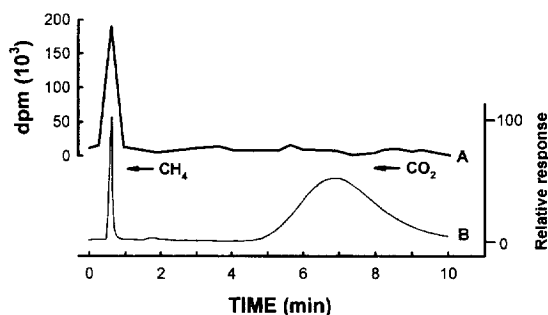


Fig. 4. Gas chromatographic analysis of the gas phase of an acetate-grown culture of *Methanosarcina thermophila* containing uniformly labeled [^{14}C]Kepone. Volatile products in the headspace of the culture described in Fig. 3A were determined after 90 days. (A) Chromatogram of a headspace sample (50 μl) from the culture; (B) chromatogram of sample containing methane and CO_2 standards.

the CO dehydrogenase enzyme complex is at least partially responsible for the conversion of Kepone by acetate-grown cultures. Conversion was not detected when titanium(III) citrate was omitted (data not shown) which suggests a reductive mechanism. Similar results were obtained with titanium(III) citrate-reduced factor III isolated from the CO dehydrogenase enzyme complex (Fig. 2). These results suggest that factor III in the Co/Fe-S component is at least one site for Kepone conversion by the CO dehydrogenase enzyme complex.

Kepone is reductively dechlorinated by reduced vitamin B_{12} to indene derivatives ($\text{C}_9\text{Cl}_8\text{-nH}_n$, where $n = 3\text{--}5$) [13]. The TLC pattern of soluble decomposition products obtained with titanium(III) citrate-reduced vitamin B_{12} (Fig. 2) was the same as that produced from Kepone by acetate-metabolizing cultures of *M. thermophila* and reduced factor III. The results suggest that acetate-grown *M. thermophila* converted Kepone to products previously reported for the dechlorination of Kepone by reduced vitamin B_{12} [13].

The metal centers of the Ni/Fe-S component of the CO dehydrogenase enzyme complex are reduced in the presence of CO; however, the CO-reduced complex converted only a trace amount of Kepone after 2 h to the nonpolar product (Fig. 2). Either the CO-reduced Ni/Fe-S component is unable to reductively convert Kepone, or Kepone inhibits CO reduction of the component. The former prospect is con-

sistent with an inability of the Ni/Fe-S component to reductively dehalogenate trichloroethylene [1]. The CO-reduced Ni/Fe-S component transfers electrons to the Co/Fe-S component reducing the bound factor III [12]; thus, the inability of CO to serve as a reductant for Kepone dechlorination by the CO dehydrogenase complex suggests that Kepone interferes with CO-dependent reduction of the Co/Fe-S component either by preventing reduction of the Ni/Fe-S component or by transfer of electrons to factor III present in the Co/Fe-S component.

Coenzyme F_{430} , a nickel(II) porphyrinoid present in acetate-grown *M. thermophila* [14], catalyzes the reductive dehalogenation of chlorinated C_1 hydrocarbons [2]. Reduced coenzyme F_{430} , isolated from *M. thermophila*, catalyzed the conversion of Kepone to the same products as did acetate-grown cultures, the reduced CO dehydrogenase enzyme complex, reduced factor III isolated from the CO dehydrogenase complex, and reduced vitamin B_{12} (Fig. 2). No conversion occurred without titanium(III) citrate suggesting a reductive mechanism for the conversion by F_{430} . The results suggest that methyl coenzyme M methylreductase is another potential site for reductive dehalogenation of Kepone by acetate-grown *M. thermophila*.

Although the mechanism and products of Kepone conversion by *M. thermophila* have not been fully characterized, the results presented here suggest that acetate-grown methane-producing anaerobes are able to reductively dechlorinate Kepone utilizing enzyme-bound factor III and possibly enzyme-bound coenzyme F_{430} .

It remains to be determined whether the products are less toxic and more susceptible to further degradation; however, previous studies show that the dechlorination of Kepone by reduced vitamin B_{12} leads to destruction of the dihomocubane structure promoting further decomposition [13].

The concentration of Kepone approaching that of contaminated James River sediments (0.02 mg/ml) inhibits many aerobic microbes from estuarine environments, although anaerobes are less sensitive [15]. The concentration of Kepone used in this study (0.79 mg/ml) had no effect on growth and methanogenesis by *M. thermophila* (data not shown) suggesting that acetotrophic methane-producing anaerobes are able to tolerate Kepone concentrations encountered

in contaminated sediments. The results presented here suggest that further studies are warranted to determine if acetotrophic methane producers can be utilized for the bioremediation of sediments contaminated with Kepone and possibly other highly chlorinated complex hydrocarbons.

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