Enhanced Biotransformation of Carbon Tetrachloride by *Acetobacterium woodii* upon Addition of Hydroxocobalamin and Fructose

SYED A. HASHSHAM1* AND DAVID L. FREEDMAN2

Center for Microbial Ecology and Department of Civil and Environmental Engineering, Michigan State University, E. Lansing, Michigan 48824, and Department of Environmental Engineering and Science, Clemson University, Clemson, South Carolina 29631

Received 2 April 1999/Accepted 19 July 1999

The objective of this study was to evaluate the effect of hydroxocobalamin (OH-Cbl) on transformation of high concentrations of carbon tetrachloride (CT) by *Acetobacterium woodii* (ATCC 29683). Complete transformation of 470 μM (72 mg/liter [aqueous]) CT was achieved by *A. woodii* within 2.5 days, when 10 μM OH-Cbl was added along with 25.2 mM fructose. This was approximately 30 times faster than *A. woodii* cultures (live or autoclaved) and medium that did not receive OH-Cbl and 5 times faster than those controls that did receive OH-Cbl, but either live *A. woodii* or fructose was missing. CT transformation in treatments with only OH-Cbl was indicative of the important contribution of nonenzymatic reactions. Besides increasing the rate of CT transformation, addition of fructose and OH-Cbl to live cultures increased the percentage of [14C]CT transformed to 14CO2 (up to 31%) and 14C-labeled soluble materials (principally L-lactate and acetate), while decreasing the percentage of CT reduced to chloroform and abiotically transformed to carbon disulfide. 14CS2 represented more than 35% of the [14C]CT in the presence of reduced medium and OH-Cbl. Conversion of CT to CO was a predominant pathway in formation of CO2 in the presence of live cells and added fructose and OH-Cbl. These results indicate that the rate and distribution of products during cometabolic transformation of CT by *A. woodii* can be improved by the addition of fructose and OH-Cbl.

Metallocoenzymes like corrinoids, cytochrome P-450, and iron(II) porphyrins are known to play a major role in biotransformation and detoxification of carbon tetrachloride (CT) (22). The homoacetogen *Acetobacterium woodii* (1) is one of many organisms that transform CT at relatively high rates due in part to its use of the acetyl coenzyme A (CoA) pathway and correspondingly high levels of corrinoids (25). CT is transformed by fructose-grown *A. woodii* to mainly chloroform (CF), dichloromethane (DCM), and several soluble nonchlorinated products (9, 10, 24). Autoclaved *A. woodii* cultures also transform CT at an appreciable rate, perhaps indicating the heat stability of the enzymes involved. CT is also transformed by *Shewanella alga* BrY, an organism lacking the acetyl-CoA pathway (28). BrY reduces vitamin B12, which subsequently recycles supplemental corrinoid to its reduced form, making it possible to monitor growth of *A. woodii* cells in CT transformation when provided with fructose and hydroxocobalamin (OH-Cbl). Based on results from a methanogenic enrichment culture (14), we hypothesized that a pure culture of *A. woodii* supplemented with fructose and OH-Cbl would transform CT faster than autoclaved cells with the same amount of OH-Cbl and faster than OH-Cbl present in sulfide-reduced basal medium. In addition, we expected the presence of live cells supplemented with fructose and OH-Cbl to shift the product distribution from CT away from CF and DCM and towards CO and CO2. The results indicate that *A. woodii* recycles supplemental corrinoid to its reduced form, increases the rate of CT transformation to CO versus carbon disulfide (CS2), and drives metabolism of CO to CO2 and fermentation products.

(See preliminary results of this study were presented at the 96th Annual Meeting of the American Society for Microbiology, New Orleans, La., 19 to 23 May 1996.)

MATERIALS AND METHODS

Chemicals. CT, CF, and DCM (all ≥99.9%) and acetate, lactate, fumarate, and isobutyrate were obtained from Aldrich Chemical Co.; CS2 was from EM Industries, Inc. OH-Cbl (98%) and cyanocobalamin (99%) were purchased from Sigma Chemical Co. [14C]CT was obtained from Dupont NEN Products and diluted to 1.9 × 107 dpm/ml with distilled deionized water (0.54 mM CT). The radiochemical purity of this [14C]CT stock solution was evaluated to be 99.6% ± 0.11% (± 1 standard deviation for duplicate bottles) by using the procedures described under “Analysis of 14C products.” EcolScint (Baker Diagnostics, Inc.) was used as liquid scintillation cocktail for all 14C radioactivity measurements.

Growth conditions. *A. woodii* (ATCC 29683) was grown on fructose in a basal medium described by Freedman and Gossett (11), modified as follows: the Fe2+ concentration was reduced to 1.2 mg/liter, and 10 ml of a vitamin solution per liter was added (27). Fructose was added as a sterile stock solution (2.8 or 25.2 mM).

*A. woodii* was grown in 160-ml serum bottles that were modified by connecting a 1-cm inside-diameter test tube at a right angle to the side of the bottles near the base, resulting in a final bottle volume of 173 ml. These modified serum bottles resemble culture flasks with a side arm (e.g., Belco Biotechnology or Ace Glass), making it possible to monitor growth of *A. woodii* by optical density at 620 nm.
(Bausch and Lomb Spectronic 20 spectrophotometer). A correlation was developed between optical density and dry weight. Cells were harvested by centrifugation and resuspended in the basal medium. The suspension was filtered through a glass fiber filter (Whatman, 21 µm, dried at 37°C, and weighed. An optical density of 0.1 using a 1-cm light path corresponded to 45 mg of cells per liter.

Basal medium was distributed to serum bottles in an anaerobic glove box (Coy Laboratory Products). The headspace was filled with 5% N2-CO2 gas mixture (70%/30% vol/vol) to remove oxygen and equilibrate the bicarbonate in the basal medium with CO2 (resulting in a pH of 7.0). The serum bottles were scaled with slotted gray butyl rubber septa, covered with aluminum foil, and purged at 35°C on a gyratory shaker table, with the liquid in contact with the septum.

**Volatile organic product analyses.** CT, CF, DCM, CH4, and CS2 were measured by gas chromatographic analysis (Perkin-Elmer model 9000) of 0.5-ml samples taken from the 65-ml headspace of the serum bottles. A Carbopack 1% SP-1000 column was used along with a flame ionization detector, as previously described (11). Although flame ionization is not the most suitable detector for measurement of CS2, a linear response was obtained for concentrations less than 50 µmol of CS2 per bottle. Detection limits (nanomoles per bottle) were 60 for CH4, 20 for DCM, 100 for CS2, 20 for CF, and 47 µmol for CT.

**CT transformation studies.** Experiments were initiated by adding 2 ml of a log-phase culture of _A. woodii_ growing on fructose to 106 ml of basal medium in serum bottles. Initially 2.8 mM fructose was added, to grow the culture to a dry cell mass of 12.2 ± 0.44 mg/bottle. During this period, the bottles were shaken. After growth was complete (i.e., no further increase in optical density) by day 3, 11 treatments were set up, using duplicate serum bottles for each treatment. CT (100 µmol per bottle), additional fructose (25.2 mM), and OH-Cbl (100 mM) per bottle were then added to appropriate serum bottles, which were monitored for optical density and CT transformation products. Treatments 1 to 7 consisted of sulfide-reduced basal medium (2.1 mM sulfide) with combinations of live or autoclaved _A. woodii_, OH-Cbl, and fructose. Water controls (treatment 8) consisted of 160-ml serum bottles with 100 ml of autoclaved deionized water and 100 µmol of CT, to evaluate losses through the septum and measurement of the radiochemical purity of the [14C]CT. Treatments 9 through 11 were included to check growth of _A. woodii_ on fructose and/or OH-Cbl.

**Analysis of 14C products.** [14C]CT was added (approximately 1.9 × 10^6 dpm) along with an equal amount of CT to two serum bottles representing treatments 1 to 8 (see Table 1). Treatment 1 (live _A. woodii_ culture plus [14C]CT plus OH-Cbl plus fructose) included two identical sets; one analyzed for 14C transformation products after the incubation period. CT transformation rates are obtained by dividing the initial 14C activity, fractions were collected in 0.5- to 1-min intervals, mixed with 15 ml of scintillation cocktail, and counted on a liquid scintillation counter. The identity of 14C-labeled 1-lactate was also confirmed by its reactivity with lactate dehydrogenase. The average percent recovery during the HPLC analysis ([2/14C in all fractions]/[14C injected]) was 98% ± 5.0%.

The recovery efficiency during the 14C analyses was defined as the total disintegrations per minute in all CT components (CT + CF + DCM + CS2 + CO + CO2 + pyruvate + 1-lactate + acetate + isobutyrate + unidentified soluble NSR + cell associated fraction) divided by the total disintegrations per minute present at the time of analysis. The total disintegrations per minute present at each bottle at the time of 14C analysis was the sum of 0.5-ml headspace sample disintegrations per minute and 100-µl aqueous phase sample disintegrations per minute measured by direct injection into scintillation cocktail. For bottles with low CS2 levels (i.e., <22%), the recovery based on 14C remaining was 96% ± 5.7%; for bottles with high CS2 levels, the recovery was 80% ± 1.2.

All 14C transformation products are expressed as percent of disintegrations per minute initially injected without correcting for losses through the septum during the incubation period. CT transformation rates are obtained by dividing the difference of initial and final aqueous CT concentrations by the number of days it took for the transformation, adjusted for losses in the water controls.

**RESULTS AND DISCUSSION**

**Rate enhancement due to OH-Cbl addition.** The focus of this research was to determine if the rate of CT biotransformation by _A. woodii_ can be enhanced by biochemical amendments, especially at high CT concentrations. OH-Cbl was selected for this purpose because cobalamins are one of the main coenzymes in the acetyl-CoA pathway implicated in the catabolic transformation of CT by _A. woodii_ (10), and they are also known to transform CT in abiotic systems (18).

Results presented in Fig. 1 demonstrate that adding OH-Cbl and fructose to _A. woodii_ caused a 30-fold increase in the rate of CT biotransformation with respect to controls that did not receive additional OH-Cbl (26 mg/liter/day for treatment 1 versus 0.83 mg/liter/day for treatment 5). When fructose or _A. woodii_ cells were omitted or _A. woodii_ cells were killed (treatments 2, 3, and 4), the addition of OH-Cbl still increased the rate fivefold, indicating the importance of abiotic sulfide-mediated CT transformations. The difference in rate between the treatment that contained _A. woodii_ with fructose and OH-Cbl and all others that received only OH-Cbl indicates that _A. woodii_ cells needed fructose to take advantage of the added OH-Cbl for CT transformation. The rate of CT transformation by _A. woodii_ receiving only fructose was similar to previously reported rates (0.8 to 1.7 mg/liter/day) by Stromeyer et al. (24, 25). The addition of OH-Cbl (1 µmol/bottle) added in this study was only 1% of the initial CT, but it substantially increased the total amount of corrinoids present. Based on a corrinoid content of 0.52 nmol/mg of dry cell mass (7) and a cell mass of 40 mg/bottle, the corrinoid contributed by _A. woodii_ cells was only 2% of the added OH-Cbl.

When OH-Cbl was replaced with an equimolar amount of cyanocobalamin in the live culture with fructose, there was only a marginal enhancement in the rate of CT transformation over live cells alone (data not shown), perhaps due to the toxicity of cyanide to _A. woodii_ (20). Cyanocobalamin was successful in improving the rate of CT and CF transformation, as reported previously in a DCM-grown enrichment culture (2, 14) as well as in a mixed culture (15).

Since biomass concentration typically has a significant effect on the rate of CT transformation, growth was also monitored in all treatments involving _A. woodii_. The presence of CT inhibited growth of _A. woodii_ (Fig. 1b). This was expected, since the aqueous CT concentration used was much higher than in most previous studies (72 versus <2 mg/liter). However, once CT was consumed and most of the accumulated CF was also transformed, (treatment 1; Fig. 1a and c), _A. woodii_ grew to a cell density that was slightly higher than the maximum cell density for the controls with no CT. The absence of...
FIG. 1. Transformation of CT (a), cell growth (b), and accumulation of chloroform (c), CO (d), and CS₂ (e) by *A. woodii* and basal medium under various treatment conditions. 1, live *A. woodii* with CT, OH-Cbl, and fructose; 2, live *A. woodii* with CT and OH-Cbl but no fructose; 3, autoclaved *A. woodii* with CT and OH-Cbl; 4, autoclaved medium with CT and OH-Cbl; 5, live *A. woodii* with CT and fructose but no OH-Cbl; 6, autoclaved *A. woodii* with CT but no OH-Cbl; 7, autoclaved medium with CT but no OH-Cbl; 8, autoclaved water with CT but no OH-Cbl; 9, *A. woodii* seed control with fructose but no CT and no OH-Cbl; 10, *A. woodii* seed control with OH-Cbl and fructose but no CT; and 11, *A. woodii* seed control with OH-Cbl but no fructose and no CT. Vertical bars represent 1 standard deviation for duplicate bottles.
growth with fructose and OH-Cbl until nearly all of the CF was consumed (day 8, Fig. 1c) may be indicative of the activity of A. woodii towards toxicity reduction. There was no indication of OH-Cbl being used as a carbon source for growth in the presence or absence of fructose during the period of this study (Fig. 1b, treatments 9, 10, and 11).

**Effect of OH-Cbl addition on volatile product distribution.** The distribution of CT transformation products is significantly influenced by experimental conditions, including the type of reducing environment, organisms present, and concentration of CT and coenzymes (5, 12, 16, 17, 26). The major intermediates observed during CT transformation are shown in Fig. 2. Egli et al. (10) presented a similar diagram based on studies with A. woodii, although it did not include CO or CS₂. CO formation from CT is known to be catalyzed by corrinoids under reduced conditions. Further biotransformation of CO yields nonhazardous CO₂ and organic acids. CS₂ is produced from CT mainly under sulfide-mediated reducing conditions. Although it is a neurotoxin (13), its presence in drinking water is not currently regulated. CF is the most common undesirable transformation product of CT under various anaerobic conditions. DCM may also accumulate to some extent, via reduction of CF. Strategies that minimize accumulation of CF, DCM, and CS₂ are of interest for application of in situ bioremediation.

Addition of OH-Cbl promoted accumulation of CO (Fig. 1d, treatment 1). Without OH-Cbl, there was no detectable CO (below 1 μmol/bottle in treatments 5, 6, and 7). The increase in CO beyond day 8 corresponded to an increase in cells with A. woodii, OH-Cbl, and fructose (Fig. 1b), following depletion of the accumulated CF (Fig. 1c). Addition of OH-Cbl also promoted the accumulation of CS₂ (Fig. 1e). In the bottles with live culture plus OH-Cbl and fructose (treatment 1), the CS₂ peaked after 2.5 days and remained nearly constant thereafter, since all of the CT was consumed. CS₂ formation from CT has been observed in other microbial studies carried out in sulfide-reduced media (6, 14). However, neither Stromeyer et al. (24) nor Egli et al. (10) reported any CS₂ formation in their studies with A. woodii in a sulfide-reduced medium.

OH-Cbl addition also had a significant effect on accumulation of CF. In other studies with A. woodii (9, 10, 24) and several anaerobic pure cultures (4, 19) without supplemental cobalamins, CF and DCM were among the major metabolites that accumulated during CT transformation. In this study also, accumulation of CF occurred in all of the treatments that received CT, but to a lesser extent when OH-Cbl and fructose were also present with A. woodii.

The fastest rate of CT transformation and the highest level of CO accumulation occurred with the fructose and OH-Cbl amended live A. woodii (treatment 1), with a correspondingly lower level of CS₂ (compared to treatments 2, 3, and 4) and CF accumulation. Thus, active metabolism of an electron donor by A. woodii in the presence of supplemental OH-Cbl shifted the transformation of CT in favor of CO and away from CS₂ and CF. The combination of live cells, metabolism of the electron donor, and OH-Cbl was necessary to affect this shift. CT transformation still occurred at a high rate with sulfide-reduced OH-Cbl, but in the absence of fructose and active cells, CS₂ became the predominant product.

### **Product distribution based on [14C]CT.** Use of [14C]CT made it possible to determine the distribution of soluble products and CO₂ originating from CT and to confirm the distribution of volatile products obtained by gas chromatographic analysis of headspace samples (Fig. 1). Table 1 shows the distribution of products from [14C]CT for each of the treatments.

#### **Table 1. Distribution of [14C]CT transformation products**

<table>
<thead>
<tr>
<th>Product</th>
<th>% of injected dpm by treatmenta</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2</td>
</tr>
<tr>
<td>CT</td>
<td>0.62 ± 0.76</td>
</tr>
<tr>
<td>CF</td>
<td>3.4 ± 0.01</td>
</tr>
<tr>
<td>CS₂</td>
<td>18 ± 1.3</td>
</tr>
<tr>
<td>CO</td>
<td>25 ± 1.9</td>
</tr>
<tr>
<td>CO₂</td>
<td>21 ± 0.44</td>
</tr>
<tr>
<td>Pyruvate</td>
<td>3.5 ± 0.09</td>
</tr>
<tr>
<td>Acetalate</td>
<td>2.5 ± 0.04</td>
</tr>
<tr>
<td>Acetate</td>
<td>2.8 ± 0.13</td>
</tr>
<tr>
<td>Isobutyrate</td>
<td>1.0 ± 0.03</td>
</tr>
<tr>
<td>Other soluble NSRb</td>
<td>0.2 ± 0.04</td>
</tr>
<tr>
<td>Cell associated</td>
<td>4.3 ± 0.84</td>
</tr>
</tbody>
</table>

a Values represent the mean ± 1 standard deviation for duplicate bottles.

b Two sets in duplicate.

c For treatments 5 through 8, NSR was not analyzed for components. The soluble fraction of treatment 1 (live A. woodii plus OH-Cbl, 13 days) also included ethanol (2.3% ± 0.05%).
in sand (15). This is in the same range as for CT (21), suggesting that distribution of cobalamin along with an electron donor throughout a contaminated area is a feasible process.

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REFERENCES


FIG. 2. Pathways for biotransformation of CT by *A. woodii* in sulfide-reduced basal medium. Solid arrows represent major processes observed in this study; dashed arrows represent pathways reported elsewhere, but were minor in this study. Further reduction of DCM to chloromethane is possible, but occurs at very slow rates. Numbers in parentheses represent oxidation states.


