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Biodegradation and Biotransformation of Groundwater Pollutant Mixtures by *Mycobacterium vaccae*

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Mycobacterium vaccae **can catabolize a number of major groundwater pollutants. When added singly, acetone, cyclohexane, styrene, benzene, ethylbenzene, propylbenzene, dioxane, and 1,2-dichloroethylene can be catabolized by *M. vaccae*. Catabolism of a number of these chemicals was monitored by gas-chromatographic analysis. Gas-chromatographic analysis indicated that the products of benzene degradation are phenol and hydroquinone. The products of chlorobenzene and ethylbenzene degradation are 4-chlorophenol and 4-ethylphenol. The extent that some compounds were catabolized when present as mixtures was also investigated. When toluene and benzene were present concomitantly, toluene was catabolized and benzene oxidation was delayed. Although toluene promoted the degradation of styrene, a lower rate of toluene degradation occurred when styrene was present. Both 4-chlorophenol and 4-ethylphenol had an antagonistic effect on the ability of *M. vaccae* to degrade other aromatic compounds. Studies with [^{14}C]benzene indicated that *M. vaccae* can mineralize small amounts of this compound. These results suggest that components in mixtures may have a positive or a negative effect on the rates of biodegradation of other pollutants.**

The ability of microorganisms to degrade organic compounds that are recalcitrant to other forms of life has long been known (10). The contamination of groundwater by organic pollutants has been well documented (9), and the use of microorganisms to degrade these pollutants is currently being studied extensively (1, 2, 11, 13, 15, 17, 26).

Studies indicate that the major groundwater pollutant trichloroethylene can be catabolized by *Mycobacterium vaccae* (25). Other studies have confirmed that *M. vaccae* has broad cooxidative and catabolic capacities (3, 4, 14, 20). This cooxidative attack on recalcitrant molecules can result in intermediates that are more amenable to mineralization. Although much work has been done on the degradation of single pollutants by microorganisms, considerably less attention has been devoted to the degradation of mixtures of environmental pollutants (1, 2, 22). The metabolism and physiology of members of a related genus, *Rhodococcus*, have been reviewed by Finnerty (12). The present study describes the catabolic activity of *M. vaccae* with regard to 11 groundwater pollutants and the effect of intermediates on the catabolism of these pollutants and the degradative abilities of the organism.

(Preliminary reports of this work were presented previously [5, 6].)

MATERIALS AND METHODS

Organisms and growth conditions. *M. vaccae* ATCC 29678 (JOB-5) was used in this study. *M. vaccae* was grown in L-salts medium (18) with propane as a substrate (24).

Harvesting of cells. Cells were harvested by centrifugation at 27,000 × *g* for 20 min at 4°C and suspended in L-salts medium at 1 mg/ml (dry weight) for experiments.

Analytical methods. Gas-chromatographic (GC) analysis was accomplished with a Hewlett-Packard 5880A apparatus equipped with a flame ionization detector and a 30-m 0.53-mm-inner-diameter Vocol fused-silica capillary column (Supelco, Inc., Bellefonte, Pa.). The oven temperature was isothermal at 35°C for 4 min, programmed to 190°C at 4°C/min, and kept at 190°C for 2 min. Injector and detector temperatures were 240 and 250°C, respectively.

Identification of products was confirmed by mass spectrometry with a Varian Saturn II GC-ion trap detector. Electron ionization at 70 V was used to ionize the samples. The source manifold temperature was set at 220°C. Scanning was continuous at 0.5 s per scan, from 50 to 300 Da. The GC was a Varian 3400 equipped with an 8100 autosampler. The helium pressure was 12 lb/in^2. The column used was a J&W DB5 (0.25 mm by 30 m, with a 0.25-μm film thickness). The GC program was as follows: initial oven temperature, 40°C for 1 min, then 20°C/min to 230°C (hold for 0.5 min). Products were confirmed by comparison with the mass spectra of authentic standards. None of the products were present in either the starting compounds or the uninoculated controls.

Degradation of single pollutants. The ability of *M. vaccae* to degrade pollutants added singly in sealed vials was monitored by the procedure of Wackett and Gibson (26), modified as follows: 100 ppm (vol/vol) of the test pollutant was added to 100 ml of a 1-mg/ml cell suspension. Two-milliliter aliquots of this mixture were dispensed into a series of 10-ml serum vials sealed with Teflon-lined septa. Vials were incubated at 30°C on a rotary shaker at 150 rpm in an inverted position. A vial was removed at 0, 12, 24, and 48 h (the 0 h vial served as a control). After vial removal, the reaction mixture was extracted with 1 ml of ethyl ether containing 25 ppm of benzene (internal standard). One milliliter of ether extract was placed in a 1.0-ml autosampling vial and subjected to GC analysis as described above. Time-zero samples were assumed to contain 100% of the pollutant present. Later results were divided by time-zero sample results and multiplied by 100 to obtain the percentage of the compound present. In every case, uninoculated controls were monitored to ensure that the test substrate was not disappearing by abiotic means.

Degradation of multiple pollutants. In determining the catabolism of mixtures, the initial concentration was lowered to 50 ppm and 140-ml serum bottles containing 10 ml of

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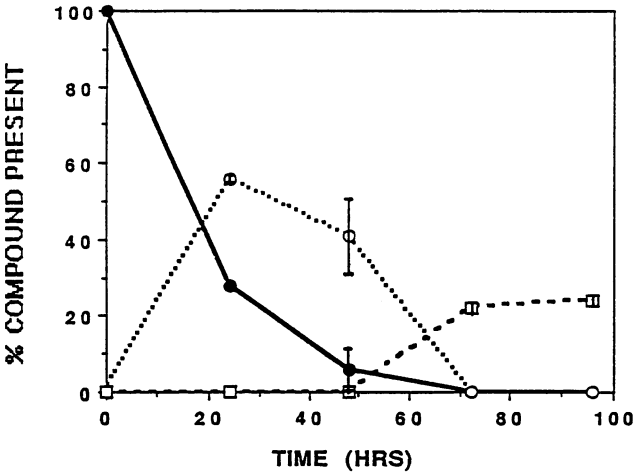

FIG. 1. Degradation of benzene at a concentration of 50 ppm by JOB-5. Symbols: ●, benzene; ○, phenol; □, hydroquinone. Bars indicate the range for duplicate samples.

a cell suspension were substituted for the 10-ml serum bottles. After incubation, the reaction mixture was extracted with 2 ml of ethyl ether containing 500 ppm of chloroform (internal standard). To test for acetone catabolism, a 0.6-ml sample was removed prior to extraction and 200 μl of 500 ppm of chloroform in H_2O was added as a standard.

^{14}C radiolabel analysis. A cell suspension was prepared as described above with 50 ppm of test pollutant. Ten-milliliter aliquots of the cell suspension were placed in 140-ml serum bottles, and 0.5 μCi of ^{14}C-labeled substrate (in ethanol) was added. The bottles were sealed and incubated as described above. The individual test bottles were removed after 24, 48, and 72 h and analyzed by the procedure of Fogel et al. (13). A 0-hour test served as a control.

RESULTS

Growth of *M. vaccae* on groundwater pollutants. *M. vaccae* can catabolize 11 major groundwater pollutants. The ability to utilize these 11 compounds as a sole source of carbon was determined. Only acetone and toluene supported the growth of *M. vaccae*. Propylbenzene, styrene, 1,2-dichloroethylene, *o*-xylene, dioxane, cyclohexane, benzene, ethylbenzene, and chlorobenzene added singly did not.

Degradation of single compounds. GC analysis indicated that both acetone and toluene at an initial concentration of 100 ppm completely disappeared in 48 h. At 100 ppm, propylbenzene, 1,2-dichloroethylene, *o*-xylene, dioxane, styrene, and cyclohexane were catabolized less than 50%. Benzene, ethylbenzene, and chlorobenzene were not measurably degraded by *M. vaccae* at 100 ppm.

For monitoring the disappearance of single compounds at 50 ppm, the procedure used for multiple pollutants was employed.

(i) Benzene. There was no noticeable degradation of benzene at 100 ppm, but at a concentration of 50 ppm (Fig. 1), the peak fell to below detectable limits in 72 h. Two product peaks appeared as benzene was degraded. A peak with a retention time equal to that of phenol appeared early and disappeared by 72 h. A second peak corresponding to hydroquinone was evident after 48 h. These peaks were identified as phenol and hydroquinone by GC-mass spectrometry.

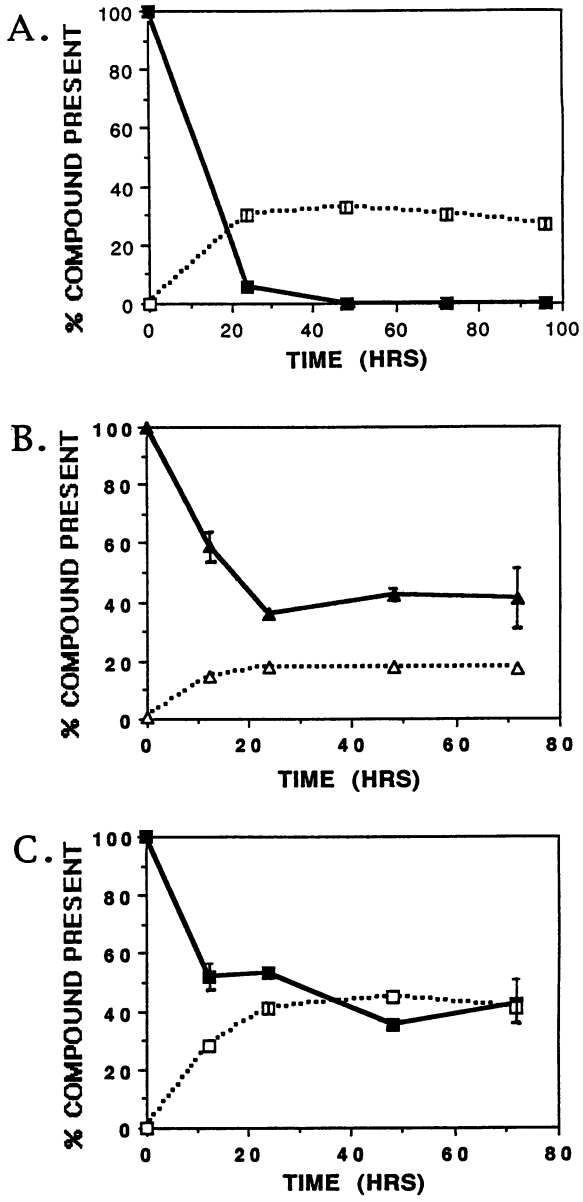

FIG. 2. (A) Degradation of propylbenzene at a concentration of 50 ppm by JOB-5. Symbols: ■, propylbenzene; □, 4-propylphenol. (B) Degradation of ethylbenzene at a concentration of 50 ppm by JOB-5. Symbols: ▲, ethylbenzene; △, 4-ethylphenol. (C) Degradation of chlorobenzene at a concentration of 25 ppm by JOB-5. Symbols: ■, chlorobenzene; □, 4-chlorophenol. Bars indicate the range for duplicate samples.

(ii) Chlorobenzene. *M. vaccae* degraded 65% of chlorobenzene in 48 h (Fig. 2). A product peak identified as 4-chlorophenol appeared as chlorobenzene was catabolized.

(iii) Ethylbenzene. At a concentration of 50 ppm, 80% of the added ethylbenzene was degraded (Fig. 2). A product peak identified as 4-ethylphenol appeared, and a small amount of *sec*-phenethyl alcohol was also detected.

(iv) Propylbenzene. Propylbenzene at 50 ppm was catabo-

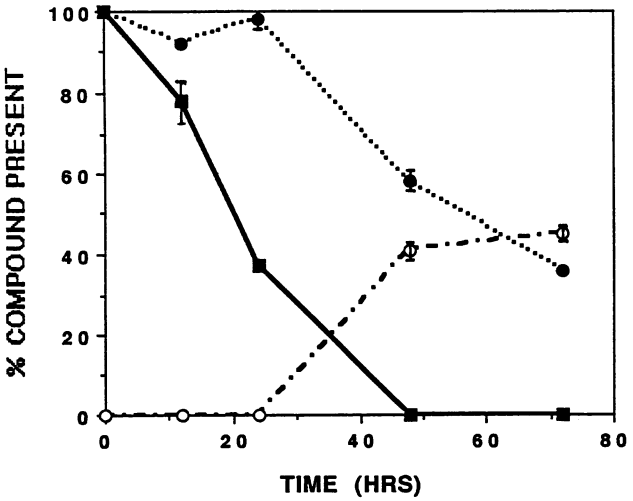

FIG. 3. Degradation of toluene and benzene, each at a concentration of 50 ppm, by JOB-5. Symbols: ■, toluene; ●, benzene; ○, phenol, a product of benzene degradation. Bars indicate the range for duplicate samples.

lized completely to 4-propylphenol and a small amount of styrene (Fig. 2).

Degradation of ^{14}C-labeled pollutants. End products of benzene and toluene catabolism were determined by use of ^{14}C-labeled substrates. Sodium [U-^{14}C]acetate was used to validate the radiolabeling assay used in this study. The results demonstrated that *M. vaccae* converted 94% of added sodium [U-^{14}C]acetate to CO_2 (81%) and cellular material (13%). About 6% of the label was present in the supernatant after incubation.

Analysis of a 0-h control indicated that under equivalent test conditions, added [U-^{14}C]toluene appeared in the supernatant (25%), headspace (61%), and CO_2 trap (4%). After incubation in the presence of *M. vaccae* for 48 h, 60% of the radioactivity was present as CO_2, 7% was present as cellular constituents, 25% remained in the supernatant, and 8% was present in the headspace.

Analysis of a 0-h control for [U-^{14}C]benzene under test conditions indicated that [U-^{14}C]benzene appeared in the supernatant (27%), headspace (68%), and CO_2 trap (4%). After incubation with *M. vaccae* for 48 h, 94% of the benzene was present in the supernatant and 6% was present as CO_2. When the initial benzene concentration was decreased from 50 to 5 ppm, an increase to 8% in the amount of [U-^{14}C]benzene converted to CO_2 was noted. This increase was noted in several experiments.

Inhibition by another pollutant. When benzene and toluene were present at equimolar concentrations, the degradation of benzene was delayed, but toluene degradation proceeded in a manner similar to that observed when toluene was added singly (Fig. 3).

Cometabolism of styrene. Styrene was catabolized 20 to 25% when added singly (Fig. 4). When styrene (50 ppm) was present concomitantly with toluene, it was totally catabolized to styrene oxide in 72 h (Fig. 4). However, the rate of toluene disappearance was slowed by the presence of styrene (Fig. 4).

Inhibition of degradation because of product formation. During the biodegradation of chlorobenzene, 4-chlorophenol was formed (Fig. 5). The formation of 4-chlorophenol inhibited the degradation of toluene (Fig. 5). Similar results were

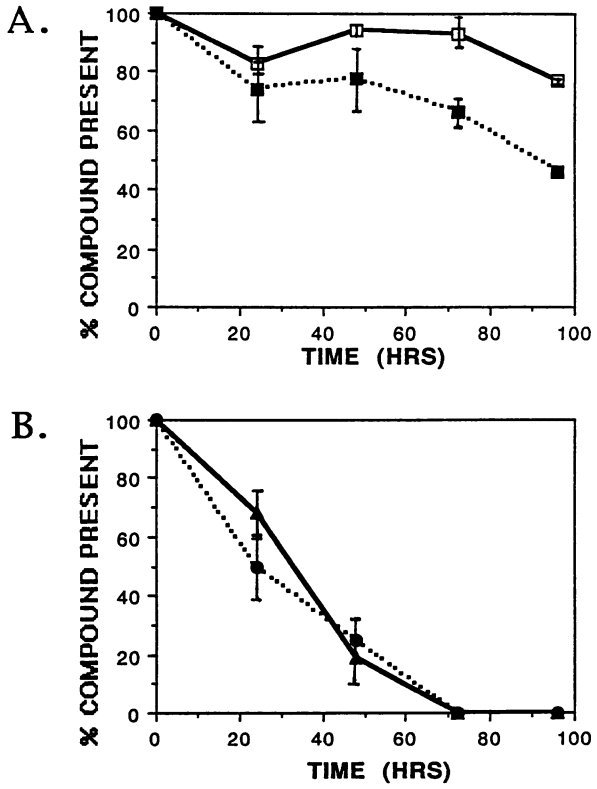

FIG. 4. (A) Degradation of styrene added singly by JOB-5. Symbols: ■, styrene; □, styrene control. (B) Degradation of styrene and toluene added concomitantly by JOB-5. Symbols: ●, styrene; ▲, toluene. Bars indicate the range for duplicate samples.

obtained for the concomitant degradation of chlorobenzene and benzene by *M. vaccae* (Fig. 5). These results were confirmed by examining the ability of *M. vaccae* to catabolize toluene or benzene in the presence of 50 ppm of 4-chlorophenol. The production of 4-ethylphenol from ethylbenzene had an equivalent effect during benzene and toluene catabolism (data not shown).

DISCUSSION

The results of this study demonstrate that *M. vaccae* can catabolize at least 11 groundwater pollutants. Only acetone and toluene support growth.

This study confirms that the major products of benzene oxidation are phenol and hydroquinone. Both phenol and hydroquinone have been implicated as biologically active toxic metabolites of benzene oxidation (23). The production of these intermediates during the degradation of benzene may be a concern. Phenol apparently is a transient intermediate, but hydroquinone is an end product. The accumulation of the potential toxic compound hydroquinone could lead to concentrations that would inhibit the degradation of other pollutants. The identification of phenol and hydroquinone as products of benzene oxidation suggests that *M. vaccae* catabolizes benzene by a pathway observed previously for mammals (8). Other studies have shown that bacteria, including a *Mycobacterium* species, metabolize benzene to catechol (7, 19). It appears that *M. vaccae* is capable of mineralizing small amounts of benzene to CO_2. Whether this mineralization takes place through phenol and

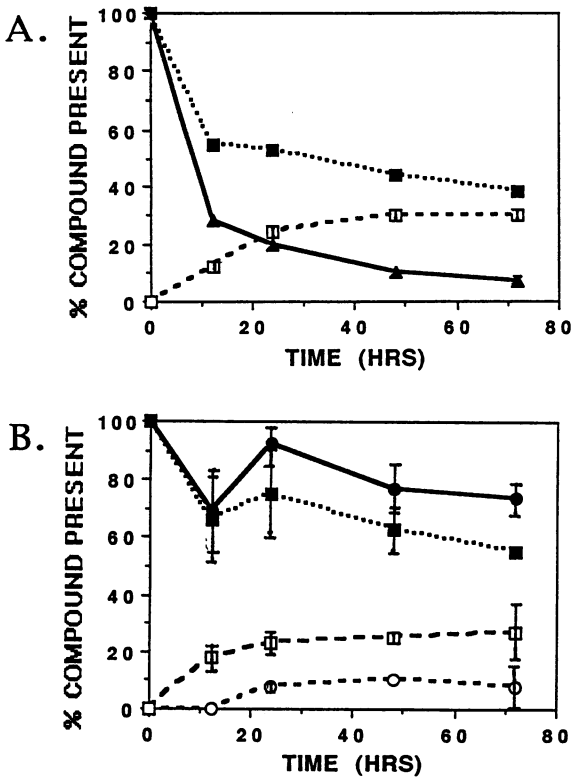

FIG. 5. (A) Degradation of toluene and chlorobenzene added concomitantly by JOB-5. Symbols: ▲, toluene; ■, chlorobenzene; □, 4-chlorophenol. (B) Degradation of benzene and chlorobenzene added concomitantly by JOB-5. Symbols: ●, benzene; ■, chlorobenzene; □, 4-chlorophenol; ○, phenol. Bars indicate the range for duplicate samples.

hydroquinone or via the catechol pathway is at this time unknown.

Antagonistic and stimulatory effects were evident when mixtures of substrates were exposed to *M. vaccae*. When toluene and benzene were present in equimolar amounts, a slight delay in the degradation of benzene was detected. This delay probably reflects the preference of the enzyme system for toluene. Styrene is cooxidized (21) to styrene oxide during toluene oxidation. The ability of a *Mycobacterium* species to oxidize styrene to styrene oxide has been reported (15, 16). It is apparent that both 4-chlorophenol and 4-ethylphenol have an antagonistic effect on the ability of *M. vaccae* to degrade other groundwater pollutants, including benzene and toluene. These compounds may exert this effect by inhibiting the enzyme responsible for the oxidation of aromatic compounds or by adversely affecting cell viability (11). The presence of inhibitory intermediates has important implications, as it could be rate limiting in the biodegradation of mixed groundwater pollutants. Further research will be directed towards identifying the mechanism of action of these antagonistic products.

Previous studies indicated that mixtures of pollutants can be catabolized to products that are either antagonistic or increase the biodegradative abilities of an organism (22). This study demonstrated that *M. vaccae* catabolizes many groundwater pollutants to more water-soluble compounds. These compounds may be more amenable to biodegradation but may adversely affect the mineralization of pollutants.

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