

Degradation of 2-sec-Butyl-4,6-Dinitrophenol (Dinoseb) by *Clostridium bifermentans* KMR-1†

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A strain of *Clostridium bifermentans*, KMR-1, degraded 2-sec-butyl-4,6-dinitrophenol (dinoseb) to a level below the limit of detection by high-performance liquid chromatography (0.5 mg/liter) within 96 h, with no accumulation of aromatic intermediates. KMR-1 could not utilize dinoseb as a sole carbon or energy source, and degradation occurred via cometabolism in the presence of a fermentable carbon source. KMR-1 mineralized some dinoseb in anaerobic cultures, evolving 7.2% of the radioactive label in U-ring ¹⁴C-labeled dinoseb as ¹⁴CO₂. The remaining anaerobic degradation products were incubated with aerobic soil bacteria, and 35.4% of this residual radioactive label was evolved as ¹⁴CO₂. During this mineralization experiment, 38.9% of the initial label was evolved as ¹⁴CO₂ after both anaerobic and aerobic phases. This is the first demonstration of dinoseb degradation by a pure microbial culture.

Dinoseb, a highly toxic herbicide, was extensively used in the Pacific Northwest until it was banned by the U.S. Environmental Protection Agency in 1986 (2, 5, 9, 24). Dinoseb does not ordinarily persist in the soil (4), but in storage areas or locations where it has been spilled, it persists as a soil (15, 23) and groundwater (6, 17) contaminant. In August 1990 the Environmental Protection Agency banned the burying of dinoseb-contaminated soils in Environmental Protection Agency-approved landfills (27), making incineration the only Environmental Protection Agency-approved disposal method for dinoseb-contaminated soil. Incineration is expensive and incomplete, leaving a noncombustible residue as a further hazardous waste and some combustion products that could remain toxic (3). Therefore, an ex situ soil bioremediation process, known as the SABRE process, that uses an anaerobic consortium and supplemental carbon source, was developed (14) and used at the field scale (12, 19, 20, 25) to successfully remediate both dinoseb- and 2,4,6-trinitrotoluene-contaminated soils (7, 12, 19, 20, 25, 26). Because of the difficulty of injecting an unisolated soil consortium into contaminated groundwater, a pure culture that degrades dinoseb could be useful for in situ aquifer bioremediation. We have isolated such a culture, *Clostridium bifermentans* KMR-1. KMR-1 was unable to use dinoseb as a sole carbon and energy source. With supplemental carbon sources, degradation to a level below the limit of detection (0.5 mg/liter) without accumulation of aromatic intermediates was observed in both anaerobic serum bottles and open, but anoxic, flasks. Mineralization experiments showed the conversion of uniformly ring-labeled [¹⁴C]dinoseb to ¹⁴CO₂ during degradation by KMR-1. This strain appears to be an excellent candidate for promoting in situ remediation of dinoseb and 2,4,6-trinitrotoluene in contaminated aquifers by an in situ SABRE process.

Bacterial strain and culture media. *C. bifermentans* KMR-1 (U.S. patent 5,455,173) was isolated from an anaerobic digester that was fed munitions compounds as its sole carbon and energy source (8, 18). KMR-1 was maintained in an oxygen-free brain heart infusion (BHI; Difco) medium prepared

anaerobically at a concentration of 37 g/liter of deionized water by bringing the medium to a rapid boil and cooling it on ice while sparging it with nitrogen gas. Cultures were incubated in sealed bottles without shaking at 28°C in the dark. Spore suspensions of *C. bifermentans* KMR-1 were used for inoculation in most experiments.

Experimental conditions. Dinoseb degradation was examined in 125-ml serum bottles containing 100 ml of oxygen-free BHI medium at a concentration of 37 g/liter in 50 mM phosphate buffer (pH 7.0) and supplemented with 100 mg of 99% pure dinoseb per liter (11). Bottles were inoculated with 0.5 ml of a heat-shocked spore suspension and incubated at 28°C in the dark. Cell growth and dinoseb degradation were monitored by high-performance liquid chromatography (HPLC).

To determine whether KMR-1 could utilize dinoseb as a sole carbon and energy source, experiments were conducted with an oxygen-free defined minimal medium containing 50 mg of dinoseb per liter and the following compounds (values in parentheses are concentrations in grams per liter): K₂HPO₄ · 3H₂O (4.2), NaH₂PO₄ · H₂O (1.0), NH₄Cl (2.0), nitrotri-acetic acid (0.1), MgSO₄ · 7H₂O (0.2), FeSO₄ · 7H₂O (0.012), MnSO₄ · H₂O (0.003), ZnSO₄ · 7H₂O (0.003), and CoSO₄ (0.001). Culture bottles were run in triplicate, and 0.5 ml of either vegetative cells or heat-shocked spores was used to inoculate each bottle. Vegetative cells were obtained from an overnight culture of KMR-1 grown in oxygen-free BHI medium and incubated in the dark at 28°C. Samples for HPLC analysis were taken periodically to monitor dinoseb degradation.

Several supplemental carbon sources were tested to find an inexpensive but comparable alternative to BHI. Molasses and solid wheat starch (supplied by Zuzanna Czuchajowska, Washington State University) at final concentrations of 1 g/100 ml in 50 mM phosphate buffer (pH 7.0) were used in separate experiments, with BHI medium used as a control at a concentration of 3.7 g/100 ml in 50 mM phosphate buffer (pH 7.0). All three media, made oxygen-free as described above, contained 50 mg of dinoseb per liter. In triplicate experiments, 125-ml serum bottles containing 100 ml of medium were inoculated with 0.5 ml of a heat-shocked spore suspension of KMR-1 and incubated in the dark at 28°C. Controls containing 100 ml of reduced 50 mM phosphate buffer (pH 7.0) without added

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carbohydrates but with 50 mg of dinoseb per liter were inoculated with 0.5 ml of a heat-shocked spore culture and incubated in the dark at 28°C. Dinoseb degradation was monitored by HPLC.

To determine dinoseb degradation rates under nonsterile conditions approximating field conditions, experiments were conducted in triplicate in 500-ml Erlenmeyer flasks containing 400 ml of 50 mM phosphate buffer (pH 7.0) with 1 g of a supplemental carbon source (wheat extract, rye extract, or molasses) per 100 ml and 50 mg of dinoseb per liter. Flasks were inoculated with 0.5 ml of a heat-shocked spore culture of KMR-1, covered with aluminum foil, and incubated in the dark at 28°C with periodic swirling to ensure a homogeneous mixture. Aqueous samples were taken and analyzed by HPLC to monitor dinoseb degradation. The two sets of controls consisted of 500-ml Erlenmeyer flasks containing 400 ml of phosphate buffer (pH 7.0) and 50 mg of dinoseb per liter. One set was inoculated with 0.5 ml of a heat-shocked spore suspension of KMR-1, and the second set was amended with 1 g of molasses per 100 ml but not inoculated. Both sets were incubated in the dark at 28°C.

Anaerobic ^{14}C dinoseb mineralization studies. Cultures containing BHI and U-ring ^{14}C -labeled dinoseb (11) were inoculated with KMR-1 to determine product mass balances and the possible production of $^{14}\text{CO}_2$. Triplicate cultures were prepared in autoclaved 125-ml serum bottles to which 100 ml of filter-sterilized BHI (3.7 g/100 ml in 50 mM phosphate buffer, pH 7.0) was added. U-ring ^{14}C -labeled dinoseb was then added at a concentration of 50 mg/liter, giving 4,200 dpm/ml. Three separate incubation conditions were tested. The first set of bottles received 0.5 ml of a heat-shocked spore suspension of KMR-1, while the other two sets served as control cultures, one set receiving 0.5 ml of an autoclaved spore suspension of KMR-1 and the second set serving as an uninoculated control. All bottles were incubated at 28°C in the dark. The $^{14}\text{CO}_2$ was periodically flushed from each bottle with sterile nitrogen gas, and radioactivity was counted in a liquid scintillation counter. The cultures were sacrificed to determine the fate of the remaining radioactive label. Culture samples were taken to determine ^{14}C uptake into cellular debris, and a sample was acidified to pH 1 with H_2SO_4 to remove and count any $^{14}\text{CO}_2$ dissolved in the medium. One culture was used for an aerobic mineralization study to determine if aerobic soil organisms could further mineralize the degradation intermediates produced during the anaerobic portion of this experiment.

Aerobic ^{14}C dinoseb mineralization studies. A 100-ml culture used in the initial anaerobic degradation of dinoseb was mixed with 100 g of sieved agricultural soil (2-mm screen) in a 250-ml Erlenmeyer flask which was placed into a heated cabinet (35°C). CO_2 -free air was sparged into the culture flask for 1 h per day. Radioactive CO_2 was trapped and counted. The culture was acidified to pH 1 with H_2SO_4 and sparged with air for 1 h to remove, trap, and then count any dissolved $^{14}\text{CO}_2$ in the medium. ^{14}C in the supernatant sample and soil extraction sample was also counted.

Analyses. HPLC (Hewlett-Packard model 1090 series II chromatograph) analysis utilized acetonitrile as the mobile phase in a filtered lithium phosphate buffer gradient made by adding 100 μl of concentrated phosphoric acid to 1 liter of deionized water and bringing the solution to pH 4.0 with 1 M lithium hydroxide. A gradient of 35% acetonitrile and 65% lithium phosphate was maintained for the first minute of the run. Over the next 15 min the gradient was increased to 100% acetonitrile and maintained for 3 min and then was returned to 35% acetonitrile and 65% lithium phosphate over the next 2 min and maintained for 2 min more. Analytes were separated

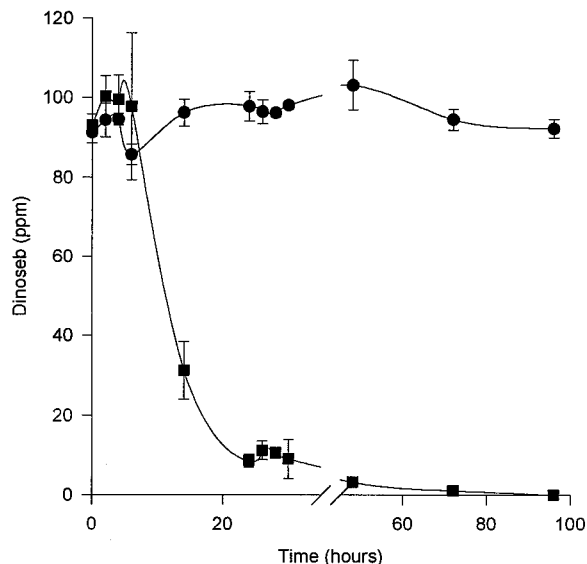


FIG. 1. Degradation of dinoseb by *C. bifermentans* KMR-1. Data are from anaerobic cultures containing BHI medium (Difco) inoculated with 0.5 ml of a heat-shocked spore suspension and incubated at 28°C in the dark. ■, BHI added; ●, BHI omitted. Each error bar shows 1 standard deviation.

on a Phenomenex Spherex 5 C_{18} column (250 by 2.0 mm) kept at 42°C throughout the run. The diode array detector was set at 254 nm, with continuous scanning of the absorption spectrum of each peak from 190 to 600 nm. The detection limit for dinoseb by this procedure was 0.5 ppm.

Anaerobic degradation of dinoseb. We have focused our work on identifying pure anaerobic isolates that can degrade dinoseb. Under optimal conditions, *C. bifermentans* KMR-1 degraded dinoseb to levels below HPLC detection limits within 96 h (Fig. 1), with no accumulation of aromatic degradation intermediates observable by HPLC. These results are similar to those seen during degradation experiments with consortia (14). Control cultures that were not amended with a supplemental carbon and nitrogen source (BHI) showed neither growth nor degradation of dinoseb. Although dinoseb degradation by bacterial consortia has been previously reported (1, 4, 12, 14, 19, 20, 22, 23), to our knowledge, this is the first report of dinoseb degradation by a pure culture. The most similar molecule seen to be degraded by a pure culture is 2,4-dinitrophenol (10, 16).

In experiments to test whether KMR-1 vegetative cells or heat-shocked spores could grow in a minimal salts medium amended with dinoseb (50 ppm), we determined that neither could utilize dinoseb as a sole carbon and nitrogen source (Fig. 2). Although clostridial spores would not be expected to germinate in a medium lacking any fermentable carbohydrates or other essential nutrients, some degradation by vegetative cells that had been transferred from a rich medium (BHI medium) was expected. Utilization of 2,4-dinitrophenol as a sole carbon and nitrogen source by strains of *Rhodococcus erythropolis* has been shown (16), raising the question of what mechanism KMR-1 uses to degrade dinoseb. Clostridia, such as *C. bifermentans*, obtain their energy and reducing power from fermentable carbohydrates or amino acids. Thus, our data showing that dinoseb degradation was probably not linked directly to the growth and the known metabolic nature of the organism make a strong case for degradation via cometabolism. Degradation of 2,4,6-trinitrotoluene and 1,3,5-triaza-1,3,5-trinitrocy-

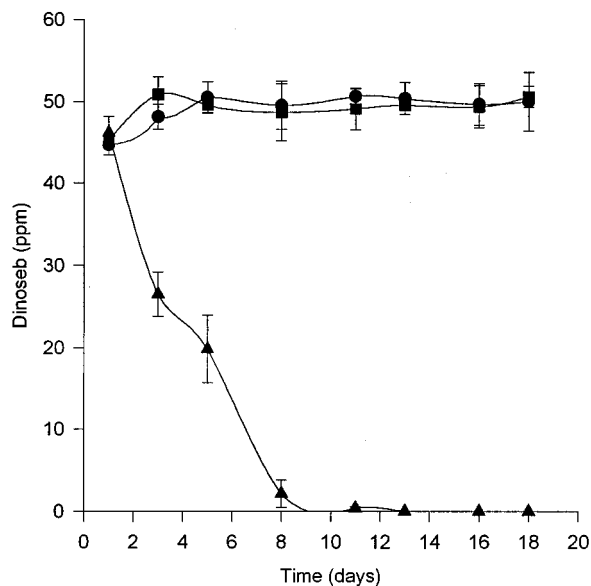


FIG. 2. Degradation of dinoseb by *C. bifementans* KMR-1 in a minimal medium. Anaerobic cultures contained minimal medium, 50 mg of dinoseb per liter, and either 0.5 ml of a heat-shocked spore culture (■) or 0.5 ml of vegetative cells (●). Incubation was at 28°C in the dark. A positive anaerobic control containing anaerobic BHI medium and 50 mg of dinoseb per liter, inoculated with heat-shocked spores, was incubated at 28°C in the dark (▲). Each error bar shows 1 standard deviation.

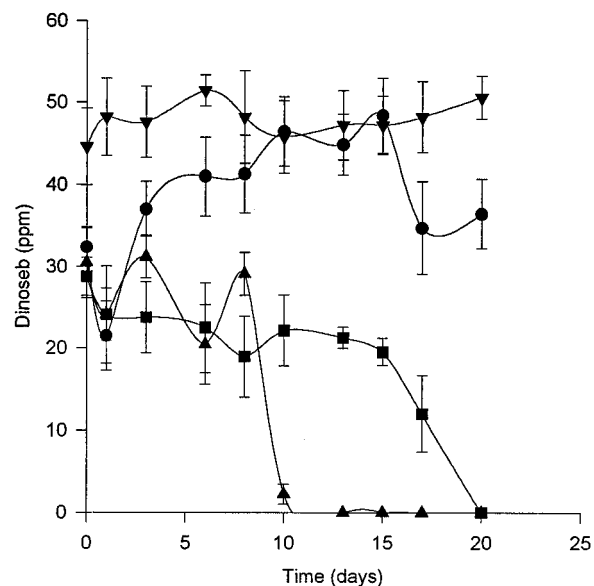


FIG. 3. Degradation of dinoseb by *C. bifementans* KMR-1 using different supplemental carbon sources. Anaerobic cultures contained phosphate buffer (pH 7.0), 1 g of either a starch extract from wheat (■) or molasses (●) per 100 ml, 50 mg of dinoseb per liter, and 0.5 ml of a heat-shocked spore culture, incubated in the dark at 28°C. Anaerobic BHI medium served as a positive control and was amended with 50 mg of dinoseb per liter and 0.5 ml of heat-shocked spores (▲). A negative anaerobic control was run in phosphate buffer with 50 mg of dinoseb per liter, and 0.5 ml of heat-shocked spores (▼). Each error bar shows 1 standard deviation.

clohexane (18) by KMR-1 was similarly cometabolic, as was that by another of our *C. bifementans* strains (21). All abiotic controls showed little to no reduction in the concentration of dinoseb during these experiments (data not shown).

Carbon source analysis. We investigated several carbon sources whose high degree of solubility in water might make them suitable for injection into aquifers along with clostridial spores. In initial experiments conducted in 125-ml anaerobic serum bottles, BHI medium was used as a control, stimulating rapid degradation of dinoseb to below the limit of detection within 11 days (Fig. 3). A soluble starch extracted from wheat, when tested as a possible supplemental carbon source, promoted complete degradation of dinoseb within 20 days (Fig. 3). However, the high solids content of the wheat extract makes it an unlikely candidate for in situ use. Molasses was also tested, but because *C. bifementans* is unable to utilize sucrose, the main carbohydrate in molasses, little if any biotic degradation or bacterial growth occurred (Fig. 3). Furthermore, molasses, which is made mostly of beet sugar, contains very little protein, about 3%. When the molasses medium was supplemented with 0.2% (wt/vol) yeast extract, growth in cultures inoculated with vegetative cells but without dinoseb occurred (data not shown). Growth on this medium, however, was very slow and quite poor compared with growth on BHI medium. Dinoseb degradation results were similar when an anaerobic consortium with yeast extract added to provide vitamins and nutrients was used (22). Our strains of *C. bifementans*, like most clostridia, require multiple vitamins and amino acids (unpublished data). This initial anaerobic experimentation showed that carbon sources other than BHI can support growth and dinoseb degradation by KMR-1 when appropriate growth factors are also provided.

The second phase of carbon source analysis was similar to those of previous studies (8, 14) with consortia that degrade dinoseb and 2,4,6-trinitrotoluene. In these mixed cultures,

anaerobiosis, induced by facultative organisms, leads to the eventual germination and propagation of the KMR-1 spores. Both wheat and rye extracts facilitated complete degradation of dinoseb within 17 days of the initial incubation (Fig. 4).

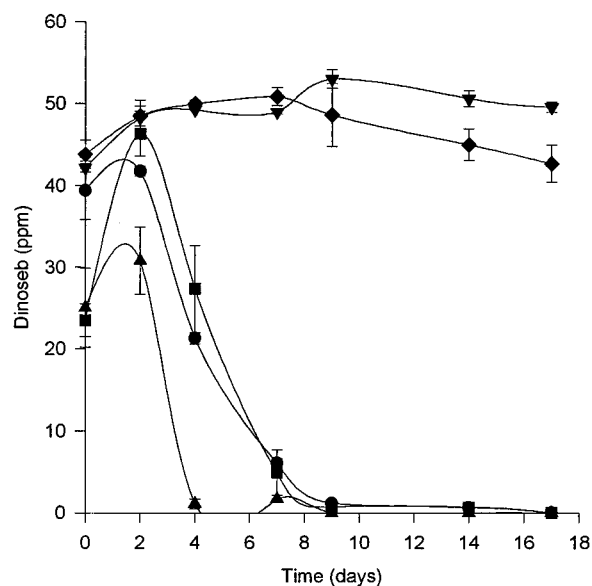


FIG. 4. Degradation of dinoseb by *C. bifementans* KMR-1, in originally aerobic mixed cultures, using different supplemental carbon sources to promote anaerobiosis. Aerobic cultures contained phosphate buffer (pH 7.0), 1 g of either a wheat extract (●), a rye extract (■), or molasses (▲) per 100 ml, 50 mg of dinoseb per liter, and 0.5 ml of a heat-shocked spore culture. Incubation was in the dark at 28°C. A second set of cultures contained molasses, but they were not inoculated (◆). Control flasks contained phosphate buffer, 50 mg of dinoseb per liter, and 0.5 ml of heat-shocked spores (▼). Each error bar shows 1 standard deviation.

TABLE 1. Mineralization of [U-ring ¹⁴C]dinoseb by combined anaerobic-aerobic processes

| Expt | Mean dpm (%) of radioactive label recovered ± SD | | | | |
|------------------------|--|---|---|--|--|
| | Initial ¹⁴ C label in supernatant | ¹⁴ CO ₂ from culture ^a | ¹⁴ C label after degradation | ¹⁴ C label in supernatant after degradation | Total ¹⁴ C label ^b |
| Anaerobic ^c | 420,705 ^d (100.0) ± 4,843 | 30,270 (7.2) ± 2,373 | 1,012 ^e (0.2) ± 507 | 376,818 ^f (89.6) ± 2,383 | 408,099 (97.0) ± 4,683 |
| Aerobic ^g | 376,818 ^h (100.0) | 133,463 (35.4) | 119,474 ⁱ (31.7) | 97,065 (25.8) | 347,002 (92.9) |

^a ¹⁴CO₂ recovered from cultures via flushing with sterile nitrogen gas and after acidification with H₂SO₄ to pH 1. Total ¹⁴CO₂ recovered from this culture was 163,733 dpm (38.9%).

^b The additive portion of the initial radioactive label that was recovered during the course of the experiment. Total ¹⁴CO₂ recovered from ¹⁴C label was 321,284 dpm (90.6%).

^c Anaerobic experiments were conducted in triplicate with [U-ring ¹⁴C]dinoseb.

^d Disintegrations per minute before inoculation with *C. bifermentans* KMR-1.

^e Cellular debris filtered, washed, and collected with a 0.22-μm-pore-size filter at the conclusion of anaerobic degradation.

^f Culture supernatant filtered through a 0.45-μm-pore-size filter at the conclusion of anaerobic degradation.

^g Aerobic experiments were conducted with the anaerobic degradation products.

^h Disintegrations per minute before inoculation with 100 g of soil.

ⁱ Radioactive label extracted from soil at the conclusion of the aerobic mineralization experiment.

Molasses promoted complete degradation within 9 days, which approaches the degradation rates seen with BHI in anaerobic serum bottles (Fig. 2) and emphasizes the importance of the aerobic and/or facultative bacteria in the initial stages of this process. A control that contained molasses but was not inoculated with KMR-1 showed no degradation, even though aerobic microorganisms reduced the redox potential to -111 mV.

Anaerobic and aerobic [¹⁴C]dinoseb mineralization. The initial average number of disintegrations per minute was 420,705 dpm per flask (Table 1). Of the initial label, 7.2% was evolved as ¹⁴CO₂ (Table 1). The resultant anaerobic culture broth was then filtered to collect and count ¹⁴C in the cell debris and aqueous phase. Control experiments with both killed and noninoculated cultures showed no growth or degradation of dinoseb throughout the anaerobic phase of this experiment.

A second experiment was conducted to determine if aerobic soil bacteria could utilize the dinoseb degradation products left after the anaerobic phase of the experiment. During the aerobic phase, 35.4% of the residual 376,818 dpm of radioactive label was evolved from the culture flask as ¹⁴CO₂ (Table 1). The combined data from both anaerobic and aerobic experiments showed that 38.9% of the initial 420,705 dpm of radioactive label was accounted for as ¹⁴CO₂ (Table 1). Similar ¹⁴CO₂ recoveries were found in [¹⁴C]dinoseb mineralization experiments in which manure was used as the inoculant (4).

While mineralization of dinoseb by an aerobic soil consortium apparently does not occur (1), aerobes easily mineralized the anaerobic degradation products of dinoseb. Stevens et al. (22) determined that after 8 weeks of incubation with an anaerobic consortium, 68.2% of the radioactive label from [¹⁴C]dinoseb resided as [¹⁴C]acetate within the cultures. A proposed pathway (13) for the anaerobic degradation of dinoseb suggests initial mechanisms by which KMR-1 degrades dinoseb to products that might be readily dearomatized in further reactions.

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