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Mineralization of the Dibenzothiophene Biodegradation Products 3-Hydroxy-2-Formyl Benzothiophene and Dibenzothiophene Sulfone

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Dibenzothiophene is degraded to 3-hydroxy-2-formyl benzothiophene by various bacteria, including a strain of *Pseudomonas putida* that also forms dibenzothiophene sulfone via an alternate pathway. By using these end products as substrates, mixed enrichment cultures that could degrade 3-hydroxy-2-formyl benzothiophene and dibenzothiophene sulfone with the formation of CO_2 were established.

Previous studies have shown that dibenzothiophene (DBT) is biodegraded aerobically by various bacteria to 3-hydroxy-2-formyl benzothiophene (3–5) and via an alternate pathway to DBT-5-oxide (4), DBT-5-dioxide (M. Mormile and R. M. Atlas, Abstr. Annu. Meet. Am. Soc. Microbiol. 1988, Q127, p. 304), *o,o'*-biphenol (D. Dutt, P. Saadi, S. Ciatti, and S. Krawiec, Abstr. Annu. Meet. Am. Soc. Microbiol. 1988, K112, p. 225), or biphenyl (T. E. Ward, D. H. Key, and B. E. Dinsdale, Symp. Biotechnol. Fuels Chem., 1988). No organism, however, has been shown to degrade DBT completely, and no previous studies on the fate of products that are formed as a result of DBT biodegradation have been reported. In this study we examined the degradation of 3-hydroxy-2-formyl benzothiophene and DBT-5-dioxide, which are the end products formed via an alternate pathway by a strain of *Pseudomonas putida* (Mormile and Atlas, Abstr. Annu. Meet. Am. Soc. Microbiol. 1988).

P. putida was grown in a medium consisting of 4 g of NaH_2PO_4, 4 g of K_2HPO_4, 2 g of $(NH_4)_2SO_4$, 0.2 g of $MgSO_4$, 0.001 g of $CaCl_2 \cdot 2H_2O$, 0.001 g of $FeSO_4 \cdot 7H_2O$, and 0.25 g of yeast extract per liter of distilled water (pH 7.2) to which DBT was added as a 10% solution dissolved in dimethylformamide, after the medium had been sterilized, to achieve a final DBT concentration of 0.05%. The cultures were incubated at 28°C with continuous shaking at 100 rpm. After 3 days to allow for product accumulation, the cells and particulates were removed by centrifugation at 10,000 × *g* by using a Sorvall RC-5B centrifuge and the supernatants containing DBT biodegradation products were filter sterilized by passage through 0.2-μm-pore-size Nuclepore polycarbonate filters. Portions (50 ml) of the sterilized product-containing medium were dispensed into sterile 250-ml flasks. The major products in the medium were identified by thin-layer chromatography, UV absorbance, and gas chromatography-mass spectrometry as *trans*-4-[2-(3-hydroxy)-benzothiophene]-2-oxo-3-butenoic acid (an intermediary metabolite in the 3-hydroxy-2-formyl benzothiophene pathway of DBT degradation) and the end product 3-hydroxy-2-formyl-benzothiophene.

Replicate flasks were inoculated with enrichment cultures that had been previously established on the same medium supplemented with 0.01% yeast extract. Two different enrichment cultures were used in these experiments. One enrichment culture came from soil that had a history of exposure to oil, and the other originated from sediment from Mill Creek, Cincinnati, Ohio, which is heavily polluted. Sterile controls were also included. The cultures were incubated at 28°C with shaking at 100 rpm. At 3-day intervals, starting at the time of inoculation, A_{390} and A_{480}, the characteristic absorbances of 3-hydroxy-2-formyl benzothiophene and *trans*-4-[2-(3-hydroxy)-benzothiophene]-2-oxo-3 butenoic acid, respectively, were determined. For these determinations, 5-ml samples were collected from each flask and centrifuged at 2,000 × *g* with a Dynac II centrifuge, supernatants were filtered through a Whatman 6F/A filter, and then absorbance measurements were made.

The concentration of *trans*-4-[2-(3-hydroxy)-benzothiophene]-2-oxo-3-butenoic acid decreased in both sterile controls and active enrichment cultures (Fig. 1). The kinetics of disappearance, however, differed between the sterile controls and the enrichment cultures. Disappearance occurred in the sterile controls at a linear rate, whereas the rates of disappearance in the active cultures indicated that disappearance was a function of the abiotic and biological rates of degradation and the rate of continued biological formation of this product from residual DBT. In contrast, 3-hydroxy-2-formyl benzothiophene did not disappear from the sterile flasks (Fig. 1). This compound, however, was rapidly biodegraded during the first 6 days of incubation by the microorganisms in both the soil and sediment enrichments. After 6 days the rate of degradation decreased.

In addition to the rate of substrate disappearance, determined by monitoring absorbance, mineralization to carbon dioxide was determined by the standard method described by the American Society for Agronomy (2). For CO_2 determinations, enrichment cultures were inoculated into replicate Biometer flasks (Bellco Glass, Inc.) containing a medium with the products of DBT biodegradation formed by *P. putida*. Sterile controls were also included. The sidearms of the Biometer flasks contained 10 ml of 0.1 N KOH to trap the CO_2 evolved. Periodically the KOH was withdrawn and replaced with fresh trapping solution. Saturated barium chloride (1 ml) was added to the KOH recovered from the trap, and the KOH was titrated to neutrality with 0.1 N HCl by using phenolphthalein as a pH indicator. The cumulative production of CO_2 was determined in this way.

The mineralization of 3-hydroxy-2-formyl benzothiophene to CO_2 followed the same pattern as substrate disappearance, with the greatest production of CO_2 occurring during the first 6 days of incubation and the rate of CO_2 evolution decreasing significantly during the next 6 days (Fig. 2). Although CO_2 was produced, no sulfate was released into

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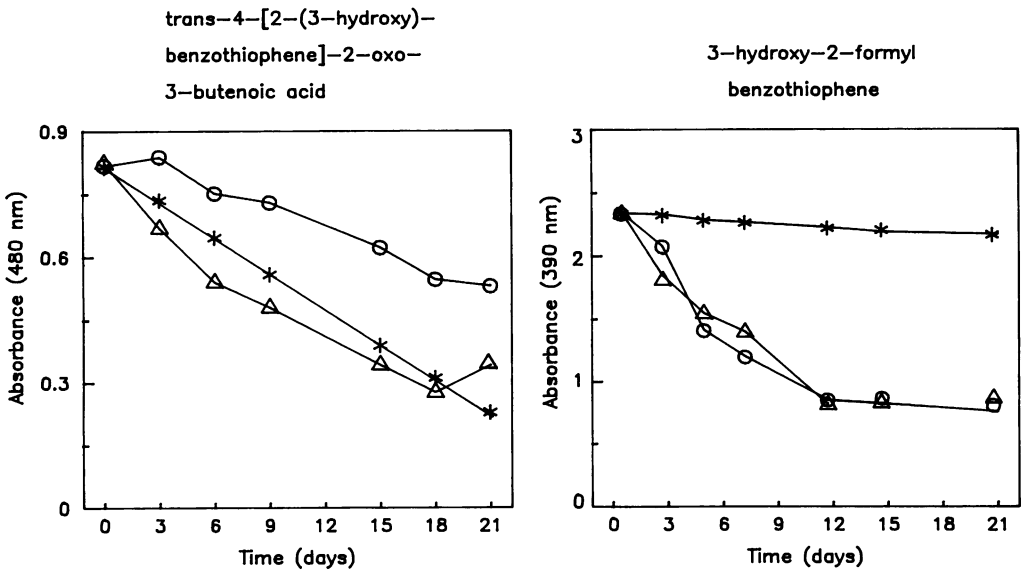

FIG. 1. Time course showing disappearance of the DBT biodegradation products 3-hydroxy-2-formyl benzothiophene and *trans*-4-[2-(3-hydroxy)-benzothiophene]-2-oxo-3-butenoic acid formed by *P. putida* by enrichment cultures. Symbols: *, sterile control; ○, soil enrichment culture; △, Mill Creek sediment enrichment culture.

the medium as determined by the standard method described by the American Public Health Association (1).

In addition to examination of the degradation of 3-hydroxy-2-formyl benzothiophene, carbon dioxide production rates were determined as follows: 50 ml of the medium used for DBT biodegradation supplemented with either 0.05% DBT-5-oxide (Aldrich Chemical Co., Inc.) or 0.05% DBT sulfone (Aldrich), the products formed by the alternate DBT degradation pathway of *P. putida*, was inoculated with the enrichments described above. Unlike in the cultures containing *trans*-4-[2-(3-hydroxy)-benzothiophene]-2-oxo-3-butenoic acid and 3-hydroxy-2-formyl benzothiophene, where there was rapid initial production of CO_2, no CO_2 evolution was found during the first 12 days with the enrichment cultures when DBT-5-oxide or DBT sulfone was used as a substrate. However, after 12 days of incubation, CO_2 evolution was observed from both DBT-5-oxide and DBT sulfone (Fig. 2). No release of sulfate was detected in these experiments.

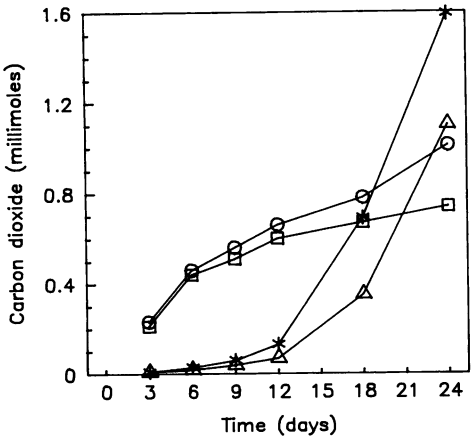

FIG. 2. Time course showing production of carbon dioxide from the DBT biodegradation products DBT-5-oxide, DBT sulfone, 3-hydroxy-2-formyl benzothiophene, and *trans*-4-[2-(3-hydroxy)-benzothiophene]-2-oxo-3-butenoic acid by enrichment cultures. Symbols: ○, soil enrichment on DBT 3-hydroxy-2-formyl benzothiophene and *trans*-4-[2-(3-hydroxy)-benzothiophene]-2-oxo-3-butenoic acid formed by *P. putida*; □, Mill Creek sediment enrichment culture on DBT 3-hydroxy-2-formyl benzothiophene and *trans*-4-[2-(3-hydroxy)-benzothiophene]-2-oxo-3-butenoic acid formed by *P. putida*; △, Mill Creek sediment enrichment culture on DBT-5-oxide; *, Mill Creek sediment enrichment culture on DBT sulfone.

Thus, we have demonstrated that the end products which have been reported to accumulate when pure cultures degrade DBT (3, 5; Mormile and Atlas, Abstr. Annu. Meet. Am. Soc. Microbiol. 1988) are subject to further degradation. However, even though carbon within the ring structure of DBT is mineralized to CO_2, we were unable to achieve release of sulfur from the thiophene ring under aerobic conditions. The evidence suggests that a succession of microbial populations is necessary to achieve complete mineralization of DBT.

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