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Selection and isolation of bacteria capable of degrading dinoseb (2-*sec*-butyl-4,6-dinitrophenol)

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Abstract

Dinoseb (2-*sec*-butyl-4,6-dinitrophenol) has been a widely used herbicide that persists in some contaminated soils, and has been found in groundwaters, causing health and environmental hazards. Persistence in some soils may stem from a lack of dinoseb-degrading organisms. We established a chemostat environment that was strongly selective for aerobic (liquid phase) and anaerobic (sediment phase) bacteria able to degrade dinoseb. The chemostat yielded five taxonomically diverse aerobic isolates that could transform dinoseb to reduced products under microaerophilic or denitrifying conditions, but these organisms were unable to degrade the entire dinoseb molecule, and the transformed products formed multimeric material. The chemostat also yielded an anaerobic consortium of bacteria that could completely degrade dinoseb to acetate and CO_2 when the *Eh* of the medium was less than -200 mV. The consortium contained at least three morphologically different bacterial species. HPLC analysis indicated that dinoseb was degraded sequentially *via* several as yet unidentified products. Degradation of these intermediates was inhibited by addition of bromoethane sulfonic acid. GC-MS analysis of metabolites in culture medium suggested that regiospecific attacks occurred non-sequentially on both the nitro groups and the side-chain of dinoseb. The consortium was also able to degrade 4,6-dinitro-*o*-cresol, 3,5-dinitrobenzoic acid, 2,4-dinitrotoluene, and 2,6-dinitrotoluene via a similar series of intermediate products. The consortium was not able to degrade 2,4-dinitrophenol. To our knowledge, this is the first report of strictly anaerobic biodegradation of an aromatic compound containing a multicarbon, saturated hydrocarbon side chain.

Abbreviations: BESA – bromoethane sulfonic acid, RAMM – reduced anaerobic mineral medium

Introduction

Dinoseb (2-*sec*-butyl-4,6-dinitrophenol) has been a widely used herbicide that, when spilled or misused, has caused health and environmental hazards [6]. It persists in some contaminated soils [15, 27], and has been found in groundwaters [6]. Persistence of the herbicide in some soils is thought to be a result of a lack of organisms capable of degrading dinoseb [27]. Dinoseb is also the major degrada-

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tion product of the herbicide Acrex by soil microorganisms [10], and is structurally similar to the herbicide 4,6-dinitro-*o*-cresol and to dinitrotoluene isomers associated with explosives manufacturing.

Dinoseb has both nitro and alkyl substituents, and microorganisms might be expected to degrade it by pathways similar to those of related compounds. Under aerobic or nitrate-reducing conditions, nitro groups can be cleaved from the aromatic nucleus to yield phenolic compounds and nitrite [26, 31]. The phenolic compounds can then be degraded by normal aromatic catabolic pathways [8]. Under anaerobic conditions, nitro groups often can be reduced to amino groups [19, 22], which are sometimes removed before further degradation of the aromatic ring [16, 32, 33]. Alkyl substituents on aromatic rings may be oxidized and subsequently degraded by β-oxidation [23]. Under anaerobic conditions, aromatic methyl groups, such as in toluene or cresol, are usually oxidized to carboxylates before ring cleavage of the resulting benzoates [24]. Larger alkyl substituents, however, have been thought to be biodegradable only if a double bond or an oxygen atom is present in the moiety [1, 23].

One laboratory has reported isolation of bacteria present at high frequency in garden soil that can grow with dinoseb as a sole carbon source [J.D. Douros, Jr., and J.J. Reid, Annual Meeting, American Society for Microbiology, 1956; and J.D. Douros, Jr., Microbial decomposition of some organic herbicides and related compounds, Ph.D. thesis, The Pennsylvania State University, 1958]. This finding has not been confirmed by subsequent researchers [11, our unpublished data]. The possibility that these organisms were growing on impurities in the culture medium and simply precipitating dinoseb must be considered. Doyle et al., however, reported $^{14}CO_2$ evolution from ^{14}C-dinoseb added to soil [4], indicating that dinoseb mineralization is possible. Another group [21] concluded that dinoseb was not biodegradable in an activated sludge reactor; however, they allowed only 18 days for acclimation, which was probably insufficient for successful microbial selection. In addition, the feed-solution of these reactors was primary municipal wastewater, which might contain nitrate in sufficient levels to inhibit dinoseb biodegradation [27]. In the bovine rumen, dinoseb has been reported to undergo reduction to its amino-derivatives [7], which are more toxic than dinoseb. Pure cultures of *Azotobacter* species can also carry out this reductive transformation as well as subsequent acetylation at the 6-amino-position [29]. In a previous study, we observed dinoseb biodegradation in some previously exposed and unexposed soils [27], but did not determine whether the dinoseb was mineralized or transformed to reactive products that might subsequently be incorporated into the soil organic matter.

The objectives of this work were to enrich for dinoseb-degrading microorganisms in order to determine what microbially mediated reactions dinoseb and related compounds might be subjected to in soil, and to obtain bacteria for use in bioremediation studies. In preliminary experiments, classic batch culture enrichment procedures failed to yield dinoseb-degrading microorganisms. However, as we report here, after extended selection in a chemostat, we enriched for and isolated soil bacteria capable of transforming and/or mineralizing dinoseb. These microorganisms will be useful in studying the environmental fates of nitro- and alkyl-aromatic compounds, and may be useful for bioremediation of contaminated soils and waters.

Materials and methods

Chemicals

Nitroaromatic chemicals (99 + % purity) were obtained from Chem Service, Inc. (West Chester, Pa.), and were used without further purification. Ring-labeled ^{14}C-dinoseb was synthesized in our laboratory from ^{14}C-phenol [9], and had a radiochemical purity of at least 96%, as assessed by high-pressure liquid chromatography (HPLC) and thin-layer chromatography (TLC) coupled with liquid scintillation counting of radioactivity in TLC bands.

Enrichment

Dinoseb-degrading microorganisms were enriched using an LH Fermentation (Hayward, Calif.) series 500 fermenter with direct-drive agitation and a one-liter vessel. Approximately 250 ml of 3-mm glass beads were placed in the bottom of the chemostat vessel to allow cell attachment and prevent washout, and to act as a soil consolidation reservoir. The chemostat thus allowed simultaneous aerated (liquid phase) and non-aerated (solid phase) enrichment conditions. The feed solution was a mineral medium containing (per liter): 272 mg KH_2PO_4, 348 mg K_2HPO_4, 5 mg NA_2SO_4, 5 mg $MgSO_4 \cdot 7H_2O$, 1 mg $CaCl_2 \cdot 2H_2O$, and 0.5 mg $FeSO_4$. This feedstock was supplemented with various carbon and nitrogen sources as indicated below. The inoculum was 250 g of a mixture of soils with dinoseb-degrading activity [27], and 50 ml of municipal sewage sludge. The fermenter was operated as a chemostat at a flow rate of 10 ml/hr (D = 0.001/hr) at pH 7 and 25 C. Feedstocks used included: 50 ppm dinoseb plus 50 ppm 2,4-dinitrophenol, the dinoseb/dinitrophenol mixture plus 1 g/l NH_4Cl, 50 ppm dinoseb plus 50 ppm phenol, and 100 ppm dinoseb plus 0.5 g/l glucose and 1 g/l NH_4Cl.

Isolation and culture conditions

Microorganisms were isolated from the vessel by repeated streaking on agar plates, using the mineral medium described above supplemented with (per liter) 50 mg dinoseb, 500 mg glucose or fructose, 1000 mg NH_4Cl, 0.5 mg $MnCl_2 \cdot 4H_2O$, 0.05 mg H_3BO_3, 0.05 mg $ZnCl_2$, 0.03 mg $CuCl_2$, 0.01 mg $Na_2MoO_4 \cdot 2H_2O$, 0.5 mg $CoCl_2 \cdot 6H_2O$, 0.05 mg $NiCl_2 \cdot 6H_2O$, 0.05 mg Na_2SeO_3, and the vitamin solution recommended by Wolin [30]. Yeast extract, when used, was added to a concentration of 0.5 g/l. For denitrifying conditions, the culture medium was supplemented with 1 g/l KNO_3, boiled under N_2 gas, and sealed in serum bottles with butyl rubber stoppers before inoculation. A reduced anaerobic mineral medium (RAMM) was used for anaerobic isolations, using the same ingredients listed above, but with 10 mg/l resazurin added as a redox indicator, 10 mg/l $NaS_2O_4 \cdot 2H_2O$ added as a reducing agent, and 1.2 g/l $NaHCO_3$. When indicated, 0.1 g/l yeast extract was added to this medium. Anaerobic cultures were grown in serum bottles and anaerobe tubes sealed with butyl-rubber stoppers, using strict anaerobic procedures [17].

Optimal culture conditions were determined using 2-factor by 3-level multidimensional experimental designs [28] for carbon concentration, nitrogen concentration, pH, and temperature. Means of dinoseb biodegradation rates for each level were plotted for each factor to estimate the optimal levels.

Utilization of other nitroaromatic substrates was determined by growing cultures in media identical to the above, but with 50 ppm of the appropriate substrate substituted for dinoseb. Samples were removed periodically with sterile anaerobic syringes and analyzed by HPLC as described below.

Identification of Gram-negative isolates was attempted using the BioLog (Hayward, Calif.) GN identification system, which consisted of testing bacteria for utilization of 95 carbon substrates and comparing the resulting pattern with a database of known bacterial patterns.

Analytical techniques

Dinoseb concentrations were determined by high performance liquid chromatography (HPLC) using a binary gradient of 10% tetrahydrofuran in methanol (solution A) and 1% acetic acid in water (solution B) on a 250 × 2 mm Phenomonex (Torrance, CA) Spherex 5 μm C18 reverse phase column. A Hewlett-Packard model 1090A instrument equipped with a diode-array detector and a computerized data system was used for the analyses. The solvent flow rate was 0.4 ml/min, and the column temperature was 40 C. The gradient program was a 10-min gradient from 60% A + 40% B to 100% A (no delay), followed by 5 min at 100% A. Dinoseb and possible transformation products were detected at 268, 225, and 385 nm, with continuous scanning of the absorption spectrum of each peak from 190 to 450 nm.

Dinoseb metabolites were extracted from cultures by centrifuging 400 ml of culture medium to remove cells, acidifying to pH 2, and extracting four times with a mixture of 20 ml acetone and 20 ml chloroform. Extracts were pooled, dried over Na_2SO_4, evaporated to dryness, resuspended in 5 ml ethyl acetate, and passed through a C18 Sep-Pak cartridge (Waters Associates, Milford, Mass.) to remove high molecular weight compounds. Extracts were concentrated *in vacuo*, then layered onto 0.6 mm silica thin-layer chromatography (TLC) plates (20 cm × 20 cm), which were developed twice with a solution of methanol/toluene (1 : 8 by volume). Separated bands of metabolites (excluding those appearing in extracts of uninoculated controls) were scraped off and eluted from the silica gel with ethyl acetate. Eluates were filtered through prewashed (ethyl acetate) Whatman # 1 filter paper and evaporated to dryness.

Mass spectrometry (MS) and gas chromatography/mass spectrometry (GC/MS) were carried out at the University of Idaho Chemistry Department, using a model VG-7070 HS mass spectrometer with electron impact ionization operated at 70 EV. Other instrument settings were: 200 mA emission current, 2.5 KU multiplier, 0.45 s/decade scan speed, 0.4 s reset, 2: 50 solvent vent delay, and 3000 resolution. For GC/MS, 0.2 μl of extract was injected (splitless injector) onto a 30 m × 0.32 mm DB-5 column (J&W Scientific, Folsom, Ca). The temperature program was to hold at 140 C for 2 min, increase at 3 C per min to reach 280 C, then hold for an additional 10 min. Accurate mass measurements were obtained with perfluorokerosene as an internal reference standard. A minimum of four scans were averaged to obtain an accurate mass, and those masses with standard deviations of less than 0.005 AMU were assumed to be accurate enough to calculate molecular formulae.

Radiochemical techniques

Anaerobic cultures were incubated with 50 mg/l dinoseb, as indicated above, but 1 μCi of U-ring ^{14}C-dinoseb, dissolved in RAMM, was added to each bottle just before inoculation. After four and eight weeks, cultures were sacrificed by acidification to pH 2 with H_2SO_4 and connected to a CO_2 trapping train consisting of a series of three serum bottles, each of which contained 10 ml of 1 N KOH. The culture flasks were agitated and flushed with N_2 gas to flush all CO_2 into the traps. Samples of culture medium were then centrifuged to remove cell debris, neutralized, and passed through a C18 Sep-Pak cartridge to remove aromatic compounds. The retentate was eluted with ethyl acetate. Aromatic compounds were thus separated into the organic extract, and nonaromatics, such as acetate, were separated into the aqueous extract. Total radioactivity in both fractions was determined by liquid scintillation counting.

Acetate concentration in the polar fraction was determined by a radiotrapping procedure employing TLC according to the method of Lynes [18]. To 1 ml of each sample, 100 μl glacial acetic acid was added. From this mixture, 100 μl was spotted onto a silica-coated glass plate. The plates were developed in a 95 : 5 solution of methyl acetate and 2.5% (aqueous) ammonium hydroxide. After drying at 110 C for 5 min, plates were sprayed with a solution of 0.1% methyl red in 95% ethanol. Single, bright-red spots were obtained. These spots were scraped into a scintillation vial, shaken with 19 ml of Bio-Safe II liquid scintillation fluid, and left overnight in the dark, at room temperature, before scintillation counting.

Results

Selection of dinoseb-degrading microorganisms

No dinoseb degradation or turbidity occurred in the chemostat during 30 days with 50 mg/l dinoseb and 50 mg/l 2,4-dinitrophenol as the sole substrates. The chemostat was operated for another 30 days with 50 mg/l dinoseb plus 50 mg/l 2,4-dinitrophenol and 1 g/l NH_4Cl, but no degradation or turbidity resulted. Again, no dinoseb degradation occurred with 50 mg/l dinoseb, 50 mg/l 2,4-dinitrophenol, and 50 mg/l phenol over 42 days, but turbidity did develop. When the chemostat was operated with 0.5 g/l glucose, 100 mg/l dinoseb, and

1 g/l NH_4Cl, turbidity developed immediately, and dinoseb degradation began after 20 days. The flow rate was then increased to 20 ml/hr (D = 0.002/hr) or another 30 days of selection.

Aerobic dinoseb-transforming bacteria

A number of bacteria that could transform dinoseb were isolated from the glucose/dinoseb/NH_4Cl-fed chemostat's effluent by repeated streaking of colonies on agar plates (see Table 1). In unshaken liquid cultures, but not in shaken cultures, the dinoseb-amended culture medium turned bright red with these strains. The red products also formed in agar pour plates, and in areas of dense growth on agar streak plates. After two to three weeks, the red color faded and a brown precipitate formed. Extracts of such cultures were examined by thin-layer chromatography and showed a continuous smear of products, with no discernible bands. This indicated polymerization of dinoseb transformation products. No growth or dinoseb degradation occurred in the absence of added sugar or yeast extract. No dinoseb transformation or degradation occurred in well-aerated cultures of these isolates.

Strains TDN-2 and TDN-5 were able to carry out the transformation of dinoseb to the red metabolite (s) under denitrifying conditions, in which case the brown pecipitate formed only slowly. Transformation by strain TDN-3 was inhibited by nitrate in both microaerophilic and denitrifying conditions. No transformation occurred in anaerobic TDN-4 cultures, but it did occur when the flasks were opened and the cultures exposed to air. Strain TDN-1 was obligately aerobic and only transformed dinoseb under microaerophilic conditions.

As shown in Table 1, these dinoseb-transforming bacteria were taxonomically diverse. The 95 substrate utilization patterns did not produce significant matches with any strains in the BioLog database (data not shown). Despite this diversity, the isolates appear to carry out similar reactions with dinoseb. Because these bacteria did not mineralize dinoseb, no further work was done with them.

Anaerobic dinoseb-degrading bacteria

Sediment from the chemostat soil reservoir was used to inoculate a strictly anaerobic medium containing 1 g/l fructose, 1 g/l NH_4Cl, and 100 mg/l dinoseb. After five weeks' incubation, the bright yellow color of the medium changed to bright orange, then faded to colorlessness as turbidity developed. Culture extracts gradually lost the characteristic aromatic UV absorption spectrum of dinoseb. This activity could be maintained in mineral medium for three or four sediment-free transfers, but not in medium containing 0.2 g/l yeast extract or 5% rumen fluid. Sediment-free dinoseb-degrading cultures could be maintained indefinitely by making three transfers in mineral medium, followed by one transfer in yeast-extract-containing medium, fol-

Table 1. Characteristics of aerobic dinoseb-transforming isolates.

Isolate	Gram reaction	Shape	Colonial[a] morphol.	Oxidase	Catalase	Facultative growth[c]	Dinoseb transformed when	
							Microaeroph.	Denitrif.
TDN-1	+	rods	R,O,P	+	+	no	yes	–
TDN-2	–	rods	I,T	+/–	+	yes	yes	yes
TDN-3	–	rods	R,T	–	+	yes	yes[b]	no[b]
TDN-4	+/–	rods	R,Y	+	+	yes	yes	no
TDN-5	+/–	cocci	S,T	+/–	+	yes	yes	yes

[a] Colonial morphologies: R = round, I = irregular, S = spreading, T = transparent, O = opaque, Y = yellow, P = produces yellow-green pigment.

[b] Dinoseb transformation in this strain was inhibited by nitrate.

[c] Could grow either aerobically by respiring oxygen or anaerobically by respiring nitrate.

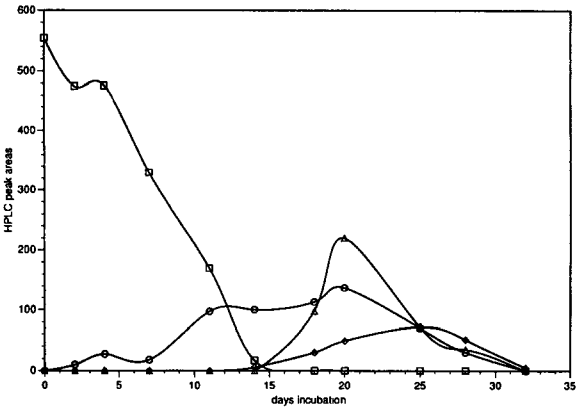


Fig. 1. Biodegradation of dinoseb (□) by the anaerobic consortium and transient accumulation of intermediate aromatic products as detected by HPLC with UV absorption detection at 268 nm. Intermediates A and B (○) coelute at 2.05 min. Intermediate C (△) elutes at 2.4 min. Intermediate D (◇) elutes at 2.7 min.

lowed by further mineral medium transfers. After 18 months of such transfers, the dinoseb-degrading cultures remained stable in the yeast-extract containing medium, which resulted in five to tenfold faster degradation of dinoseb than in mineral medium without yeast extract. Microscopic observation indicated that this stable consortium contained at least three bacterial morphologies, including short coccobacilli, 1–1.5 μm long; medium-sized rods 0.75 × 2 μm; and large rods 1–1.5 × 4 μm.

Degradation of dinoseb to nonaromatic products did not occur unless strict anaerobic procedures were followed during media preparation and culture transfers [17] and the media was prereduced to about − 200 mV. We did not establish the exact Eh required for dinoseb degradation; however, degradation did not occur when the resazurin indicator had formed a barely-visible pink color (about − 100 mV). The optimal temperature for dinoseb degradation was 30 C, although degradation occurred at temperatures as low as 15 C. Degradation was completely inhibited at 40 C. The optimal sugar concentration (fructose or glucose were equally suitable) was 0.5 g/l, while the optimal NH_4Cl concentration was 4.5 g/l; however, the culture was relatively insensitive to NH_4Cl concentration between 1 and 5 g/l.

The resulting anaerobic consortium degraded dinoseb *via* a series of aromatic products that could be detected by HPLC (Fig. 1). The UV absorption data for these intermediates are shown in Fig. 2, and can be compared with that of dinoseb. Intermediates 'A' and 'B' are shown as one line in Fig. 1, since they could not be separated by our HPLC method; however, during incubation, the UV spectrum of the peak gradually changed from the 'A' spectrum to the 'B' spectrum with time, indicating more than one component. After 30 days, no further aromatic products could be detected. A material balance for dinoseb was determined using uniformly ring labeled ^{14}C-dinoseb, and is shown in Table 2. Although a small amount of label was found in CO_2, the major product of dinoseb degradation appeared to be acetate, and the amount of label found in the acetate fraction increased with incubation time, while the amount of label in the aromatic fraction decreased.

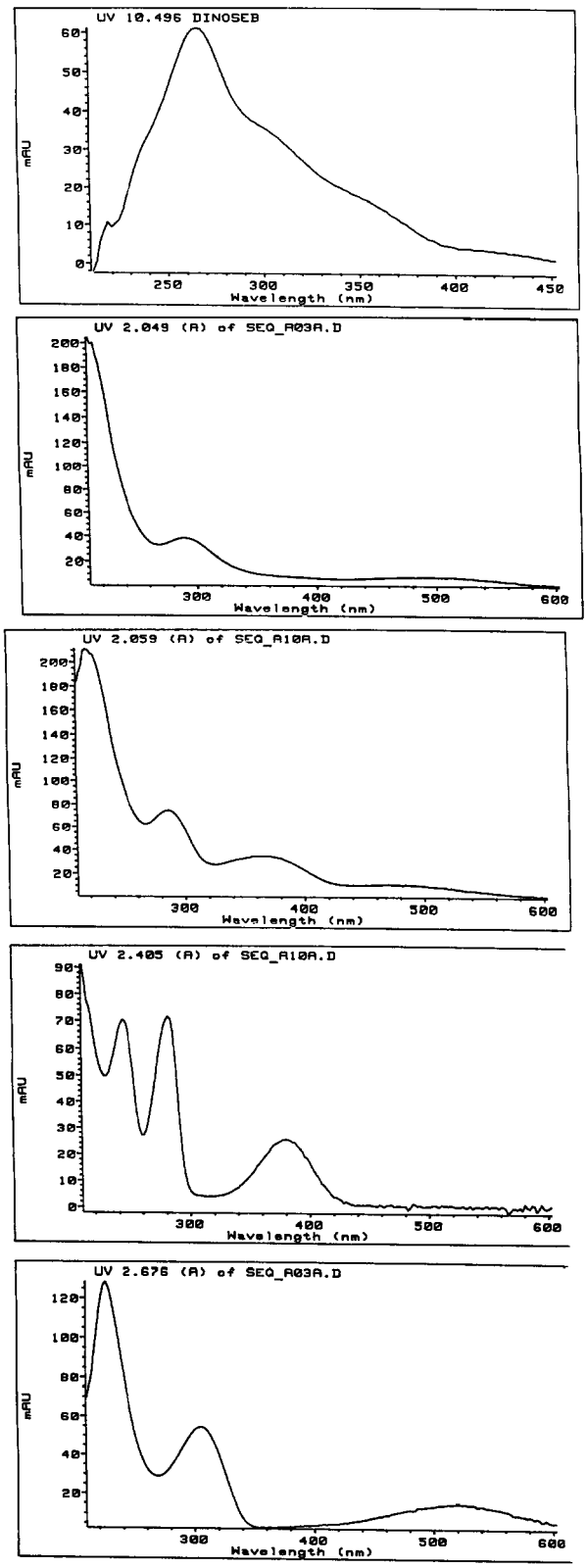
Nitroaromatic substrate range

The anaerobic consortium was tested for utilization of other nitroaromatic compounds (Fig. 3). Disap-

Table 2. Material balance for U-ring ^{14}C-dinoseb degraded by the anaerobic dinoseb-degrading consortium.

Weeks of incubation	CO_2	Percent of ^{14}C-dinoseb recovered as			
		Acetate	Aromatics[a]	Washed cells	Total recovery
4	1.2	9.6	85.8	4.6	101.2
6	0.9	24.2	74.7	2.4	102.2
8	1.2	68.2	11.3	3.3	84.0

[a] Does not contain detectable dinoseb.



pearance of parent compound was always accompanied by the appearance of a series of unidentified intermediate products (data not shown). Degradation was much slower than for dinoseb, but the anaerobic consortium was able to completely degrade 4,6-dinitro-*o*-cresol and 3,5-dinitrobenzoate to non-aromatic compounds. 2,4-dinitrotoluene and 2,6-dinitrotoluene were degraded to intermediate products, but it is unclear from this experiment whether or not the intermediates are eventually degraded to nonaromatic products. After 60 days, the concentration of 2,4-dinitrophenol began to decline, but the parent compound persisted for at least four months in these cultures. The intermediates produced from these compounds were not further characterized, but their HPLC retention times showed that different intermediate compounds were formed from different substrates.

Effect of bromoethane sulfonic acid on anaerobic dinoseb degradation

When bromoethane sulfonic acid (BESA), a specific inhibitor of methanogenesis, was added at 200 μM concentration dinoseb degradation was slowed, as shown in Fig. 4, and products C and D accumulated. These products remained in the culture medium for at least another three months.

GC-MS of culture medium

Anaerobic dinoseb cultures were extracted and subjected to TLC during both the early stages (orange color) and later stages (colorless) of degradation. Extracts from the early stages of incubation yielded two TLC bands that were not present in uninoculated controls. GC/MS analysis indicated that both bands contained multiple compounds. The mass spectra of these compounds, and of dinoseb, are shown in Fig. 5. These mass spectra showed fragmentation patterns similar to that of

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Fig. 2. UV absorption spectra of anaerobic dinoseb-degradation intermediates detected by HPLC.

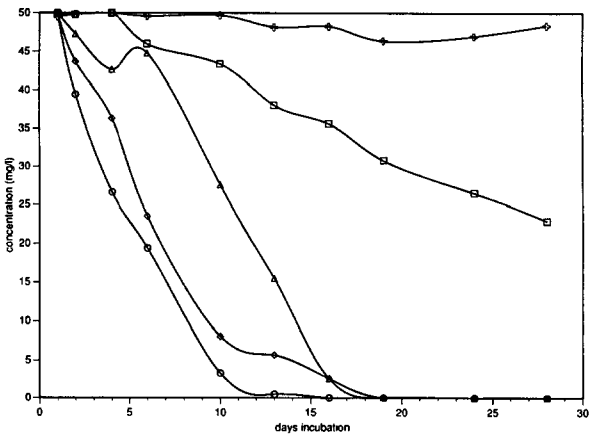

Fig. 3. Biodegradation of other nitroaromatic substrates by the anaerobic dinoseb-degrading consortium. Only disappearance of parent compounds is shown. Dinitro-o-cresol (□), 2,4-dinitrotoluene (○), 2,6-dinitrotoluene (△), 3,5-dinitrobenzoate (◇), and 2,4-dinitrophenol (✣).

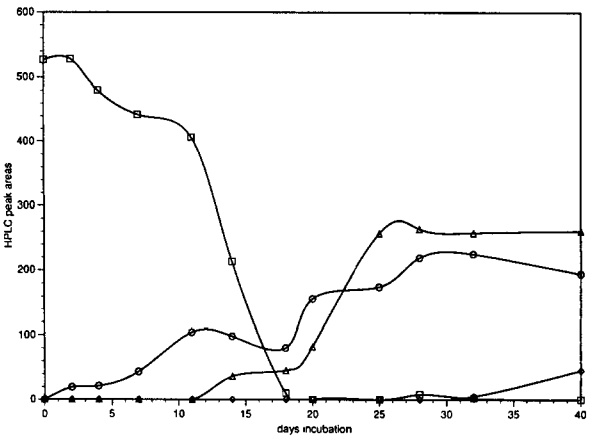

Fig. 4. Effect of 200 μM BESA on biodegradation of dinoseb by the anaerobic consortium. Compounds are as in Fig. 1.

dinoseb, confirming that they were dinoseb derivatives. These included base peaks (most abundant ions) at m/z M-29 (29 mass units less than the molecular ion), due to loss of CH_2CH_3 from the side chain [25], which indicate that the side chain is intact in these compounds, and the characteristic phenolic peak at m/z 77. The characteristic loss of 48 (NO plus H_2) from the base peak of dinoseb, characteristic of *ortho*-substituted alkyl phenols, [25] changed to a loss of 46 in these compounds.

Similar analysis of the extract from the later stages of degradation yielded one band not present in uninoculated controls, which contained two compounds (Fig. 6). These mass spectra lacked the characteristic pattern of dinoseb derivatives, but included typical aromatic patterns.

Molecular formulae, based on isotope abundance calculations from the molecular weights (± 0.005 amu), are shown in Table 3. The intermediate with molecular weight 210 could not be assigned a molecular formula from our data; however, this mass was expected from 2-*sec*-butyl-4-nitro-6-aminophenol, and the UV spectra of intermediate A (Fig. 2) was similar to that reported previously for this compound [7]. The compound with molecular weight 137 from the later stages of degradation was identified as 2-aminobenzoic acid (anthranilic acid); by GC/MS comparison with authentic standards (Fig. 6) the mass spectra of authentic 3-aminobenzoic acid and 4-aminobenzoic acid did not match this compound. Anthranilic acid did not match any of the compounds detected by HPLC of the culture medium (Fig. 1), indicating that it did not accumulate in significant quantities; however, when the culture was incubated with anthranilic acid, this compound was rapidly degraded (unpublished data). Authentic standards were not available for other putative intermediates. The extracts prepared for GC/MS analysis did not contain sufficient quantities of the compounds for HPLC analysis, hence no direct link between the compounds shown in Fig. 2 could be made.

Discussion

Chemostat selection is a means of obtaining bacteria with desired metabolic activities that cannot be isolated directly from nature through standard enrichment procedures [12, 13, 16, 20]. It is often overlooked that evolutionary time for bacteria must be measured in generations of organisms, not in years. The soil environment probably does not allow continuous growth of microorganisms, and because of this 'boom or bust' nature of growth, a bacterial cell in the soil may experience a very few generations (or none at all) in a calendar year. This can make bacterial evolution toward utilization of a

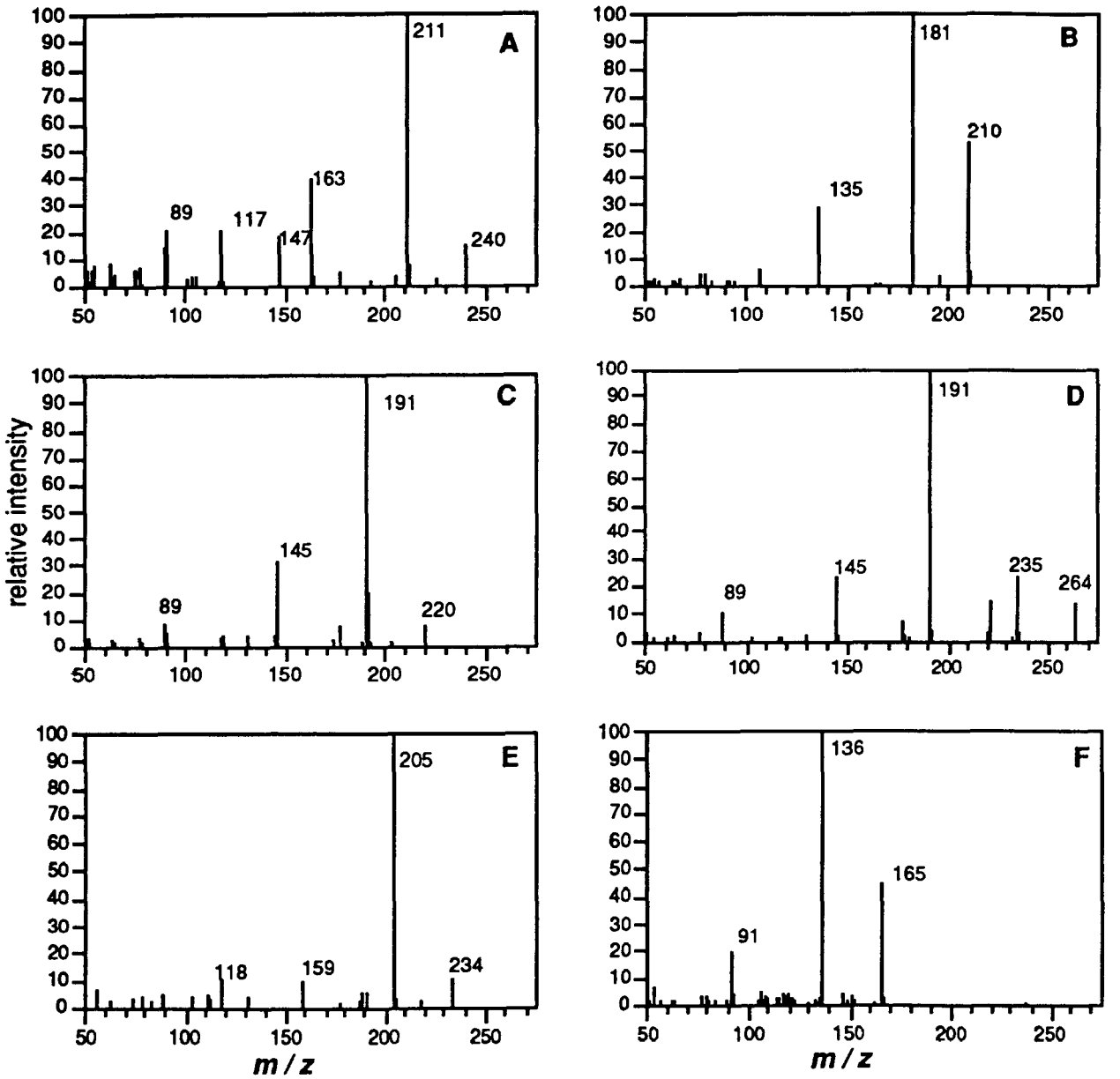

Fig. 5. Mass spectra of compounds isolated from culture medium of the anaerobic dinoseb-degrading consortium early in the incubation (medium colored orange). The y-axes are all normalized to 100% of the base peak intensity. A: dinoseb. B, C, D: from TLC band 1. E, F: from TLC band 2.

new substrate, such as dinoseb, a very slow process. By allowing profuse bacterial reproduction and simultaneously exerting strong selective pressure on the bacterial population, in a few months chemostat selection can result in 'forced evolution' that might require decades or centuries in the soil environment. In the current study, this selection method yielded dinoseb-degrading bacteria where traditional enrichment cultures did not, probably due to the constant nutrient supply to the dinoseb-degrading (or potentially dinoseb-degrading) bacteria. In batch cultures, these nutrients would have been rapidly depleted by faster-growing, non dinoseb-degrading organisms.

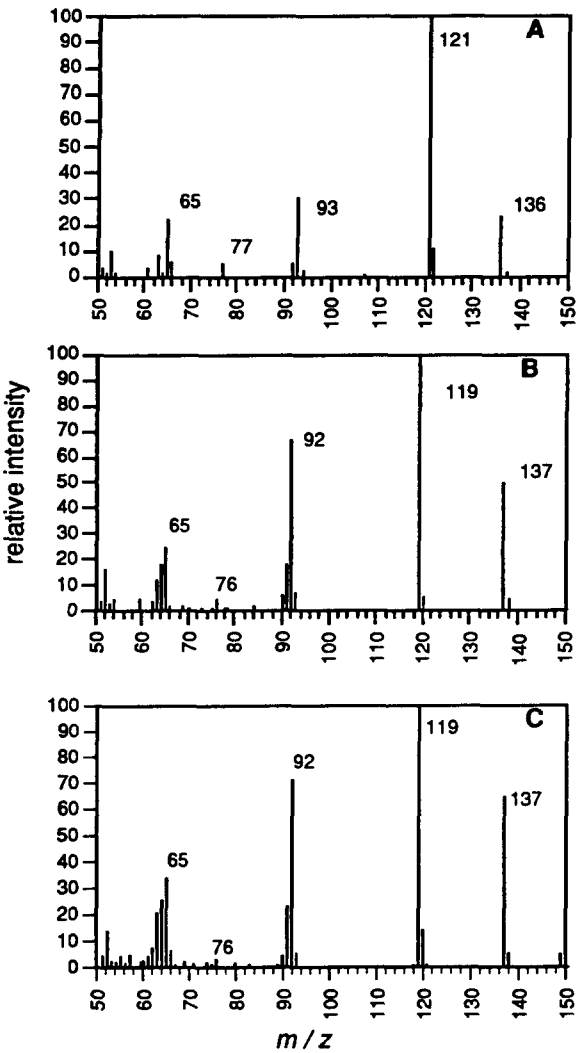

Fig. 6. Mass spectra of compounds isolated from culture medium of the anaerobic dinoseb-degrading consortium late in the incubation (medium colorless). The y-axes are all normalized to 100% of the base peak intensity. A, B: from TLC band 1. C: authentic anthranilic acid.

Reduction of aromatic nitro-groups by bacteria under anaerobic conditions [19, 22], followed by oxidative polymerization, has been described previously. The continuous smear of products on our TLC plates, along with the brown precipitate, indicate that this was probably the mode of action of the aerobic isolates. Although nitro-reduction is usually described as an anaerobic process, our observations indicate that oxygen is actually required for the reduction reactions by some strains of bacteria (strains TDN-1, TDN-3, and TDN-4). Subsequent oxidations, either nonenzymatic or mediated by oxidases [22], probably result in polymerization of the amino products. This would account for the failure to resolve discrete bands by TLC of extracts of these culture broths. Some microorganisms such as *Proteus vulgaris* [5, 14] are able to cleave bonds to release monomers from this type of polymer. Reduced derivatives of dinoseb are at least as toxic as the parent molecule [7]; thus it appears that bacteria such as these microaerophilic isolates probably do not permanently detoxify dinoseb, and might not be appropriate for use in bioremediation of dinoseb-contaminated wastewaters or soils.

Transformation of dinoseb by the diverse aerobic isolates, leading to formation of polymeric material, could indicate either that this trait is widespread in soil bacteria, or that genes for this trait were transferred, on a plasmid for example, under strong selection in the chemostat environment. Because the dinoseb-transforming phenotype was expressed under quite different conditions among the different strains (Table 1) the widespread distribution theory may be more likely.

In contrast to these reactions, strict anaerobes from the chemostat enrichment mineralized dinoseb. Under anaerobic conditions the reduced derivatives would not be subject to oxidative polymerization. Because the molecules persist as dissolved monomers, the anaerobic bacteria may have had more opportunity to adapt to their utilization than did the aerobic bacteria.

The reason that an external carbon source is required for dinoseb mineralization by the anaerobic consortium remains uncertain. One or more of these organisms may carry out alterations of the dinoseb molecule, but gain no energy from it or from subsequent products. Alternatively, microorganisms that carry out later steps in the degradation process might be inhibited by dinoseb, and thus not able to grow until the dinoseb is completely depleted. In this case, ring cleavage products would not be available for use by bacteria carrying out early steps in the degradation process.

Yeast extract appears to contain some nutrient

not present in the mineral medium that is required in small quantities for dinoseb-degradation by the anaerobic consortium. Initially, yeast extract was omitted from the media, because it appeared to allow overgrowth of the cultures by non-dinoseb-degrading bacteria; however, repeated transfers to mineral medium resulted in gradual loss of dinoseb-degrading activity. Eventually, after repeated cycling of the culture through three mineral medium transfers followed by one transfer through yeast extract-containing medium, the overgrowing organisms appeared to have been diluted out.

Inhibition of the degradation of dinoseb metabolites in the presence of BESA indicates that methanogenic bacteria participate in at least part of the process. Initial dinoseb transformation did not appear to require methanogenesis, but degradation of some intermediates did not proceed further, indicating that a hydrogen sink is probably needed to drive the final steps of degradation, possibly for thermodynamic reasons [8, 23].

Degradation of dinoseb by the anaerobic consortium appears to involve several types of biochemical reactions that have been previously described in anaerobic bacteria, and at least one reaction that has not. The initial reduction of nitro groups to amino groups appears to occur in the same manner as previously described for rumen microorganisms [7], and for *Azotobacter* species [29]. Intermediates detected with molecular weights of 220, 234, and 264 were not identified conclusively, but may represent N-alkylated forms of reduced dinoseb, similar to those produced by *Azotobacter* [29]. Anaerobic removal of an aromatic alkyl side-chain, as indicated by formation of anthranilic acid, however, has not been described previously. Degradation of multi-carbon alkyl moieties has been described under anaerobic conditions only if an unsaturated bond or an oxygenated carbon preexists in the molecule [23]. Degradation of saturated alkyl moieties should be exergonic under anaerobic conditions, but the apparent lack of such activity has been attributed to a requirement for oxygenases [23]. Our data do not contain sufficient information to assign exact structures to the intermediates without authentic standards; however, their nature can be inferred. The compound with molecular weight (MW) 210 is most likely an amino-nitro-*sec*-butyl phenol, an expected intermediate. The compound with MW 165 evidently retained the butyl side chain and one amino group, but lost the other nitrogen-containing moiety. In contrast, the compound with MW 136 had lost the butyl side chain, but retained both nitrogen moieties. These data suggest that degradation could proceed either by first removing a nitrogen-containing group or by removing the alkyl side chain (see Table 3). Simultaneous activity of different bacteria with regiospecific activities could give rise to convoluted pathways in this mixed community.

When tested with other nitroaromatic substrates, the consortium was able to completely degrade nitrocresols or nitrobenzoates, and to at least partially degrade nitrotoluenes, but was apparently not able to degrade nitrophenols. Dinoseb degradation, then, is apparently dependent on alkyl-

Table 3. Accurate mass determinations and molecular formulae for compounds detected by GC/MS.

Source	Molecular mass	Standard deviation[a]	Molecular formula	Deviation from observed mass
Dinoseb	240.0699	0.0084	$C_{10}H_{12}N_2O_5$	0.0047
Band 1	210.0891	0.0063	NR	
Band 1	220.0998	0.0081	NR	
Band 1	264.0995	0.0065	NR	
Band 2	234.0966	0.0071	$C_{12}H_{14}N_2O_3$	0.0038
Band 2	165.1147	0.0046	$C_{10}H_{15}NO$	0.0007
Band 1B	136.0600	0.0088	$C_7H_8N_2O$	0.0035
Band 1B	137.0508	0.0047	$C_7H_7NO_2$	0.0031

NR = No reasonable formula could be calculated from this mass reading.

[a] Values of 0.005 or less were considered accurate enough to define molecular formulae.

aromatic degrading bacteria, which explains why coselection with nitrophenols did not succeed in obtaining dinoseb degradation in the chemostat.

In summary, we were unable to obtain organisms that could degrade dinoseb in well-aerated cultures, and under microaerophilic or denitrifying conditions we obtained only dinoseb-transforming organisms that did not completely degrade the dinoseb molecule. We enriched and have partially characterized a strictly anaerobic consortium that degrades dinoseb completely to non-aromatic products. This consortium may be of use in bioremediation of dinoseb-contaminated sites. This is also, to our knowledge, the first report of bacteria able to remove a saturated alkyl side chain from an aromatic ring under strictly anaerobic conditions. Efforts are currently underway to positively identify intermediates in the degradation pathways, and to isolate the bacteria responsible for each reaction.

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