### ABSTRACT (Maximum 200 Words)

An oxidative pathway for the mineralization of 2,4-dinitrotoluene (2,4-DNT) by *Burkholderia* sp. strain DNT has been reported previously. We report here the isolation of additional strains with the ability to mineralize dinitrotoluene (2,6-DNT) by a different pathway. *Burkholderia cepacia* strain JS850 and *Hydrogenephaga palleronii* strain JS863 grew on 2,6-DNT as the sole source of carbon and nitrogen. The initial steps in the pathway metabolites through mass spectroscopy and nuclear magnetic resonance. 2,6-DNT was converted to 3-methyl-4-nitrocatechol by a dioxygenation reaction accompanied by the release of nitrite. 3-Methyl-4-nitrocatechol was the substrate for extradiol ring cleavage yielding 2-hydroxy-5-nitro-6-oxohepta-2,4-dienoic acid, which was converted to 2-hydroxy-5-nitropenta-2,4-dienoic acid.
Aerobic Degradation of Dinitrotoluenes and Pathway for Bacterial Degradation of 2,6-Dinitrotoluene

SHIRLEY F. NISHINO, GEORGE C. PAOLI, AND JIM C. SPAIN*

Air Force Research Laboratory, Tyndall Air Force Base, Florida 32403-5232

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An oxidative pathway for the mineralization of 2,4-dinitrotoluene (2,4-DNT) by *Burkholderia* sp. strain DNT has been reported previously. We report here the isolation of additional strains with the ability to mineralize 2,4-DNT by the same pathway and the isolation and characterization of bacterial strains that mineralize 2,6-dinitrotoluene (2,6-DNT) by a different pathway. *Burkholderia cepacia* strain JS850 and *Hydrogenophaga palmeronii* strain JS863 grew on 2,6-DNT as the sole source of carbon and nitrogen. The initial steps in the pathway for degradation of 2,6-DNT were determined by simultaneous induction, enzyme assays, and identification of metabolites through mass spectroscopy and nuclear magnetic resonance. 2,6-DNT was converted to 3-methyl-4-nitrocatechol by a dioxygenation reaction accompanied by the release of nitrite. 3-Methyl-4-nitrocatechol was the substrate for extradiol ring cleavage yielding 2-hydroxy-3-nitro-6-oxohepta-2,4-dienoic acid, which was converted to 2-hydroxy-4-nitrophenol-1,4-dienoic acid. 2,4-DNT-degrading strains also converted 2,6-DNT to 3-methyl-4-nitrocatechol but did not metabolize the 3-methyl-4-nitrocatechol. Although 2,6-DNT prevented the degradation of 2,4-DNT by 2,4-DNT-degrading strains, the effect was not the result of inhibition of 2,4-DNT dioxygenase by 2,6-DNT or of 4-methyl-5-nitrocatechol monoxygenase by 3-methyl-4-nitrocatechol.

2,6-Dinitrotoluene (2,6-DNT) and 2,4-dinitrotoluene (2,4-DNT) occur as soil and groundwater contaminants at former 2,4,6-trinitrotoluene (TNT) production sites and in the wastewater from the commercial production of feedstocks for polyurethane foam (23). Twenty years after the cessation of TNT production in the United States, the manufacturing sites are still heavily contaminated with both 2,4- and 2,6-DNT even though 2,4-DNT-mineralizing bacteria can be readily isolated from the contaminated material (26). Commercial manufacture of DNT results in the release of DNT to industrial and municipal waste treatment systems (information found at the Environmental Health Center website [http://safety.webfirst.com/eh/ew/chemical.htm] and in the TOXNET Toxics Release Inventory [http://six.nlm.nih.gov/sis1]). The unpredictable presence of DNT in the waste streams sent to the treatment plants can cause upsets in the ability of activated sludge systems to effectively remove the organic components in the waste streams (11). 2,4- and 2,6-DNT are priority pollutants (13), and industrial waste streams from DNT-manufacturing facilities are specifically regulated by the U.S. Environmental Protection Agency (40 CFR 261.32).

Contaminated munitions manufacturing sites are ready sources of bacteria able to mineralize 2,4-DNT, but bacteria able to grow on 2,6-DNT have been more elusive. The bacterial pathway for degradation of 2,4-DNT (8, 20) is initiated by dioxygenation of 2,4-DNT, which results in the formation of 4-methyl-5-nitrocatechol (4M5NC) and the release of nitrite; monooxygenation of 4M5NC then yields 2-hydroxy-5-methyquinone, which is subsequently reduced to 2,4,5-trihydroxytoluene prior to ring cleavage. The initial goal of the work was to get a sense of the distribution of bacteria able to degrade DNT. During that study, we discovered strains able to degrade 2,6-DNT and focused the rest of the work on the pathway. In order to allow rational design of bioremediation systems for 2,4-DNT-contaminated sites, it is necessary to understand the degradation of 2,6-DNT and how the degradation pathways interact.

We have examined the effects of 2,6-DNT on the degradation of 2,4-DNT by several 2,4-DNT-degrading strains. We also report here the isolation of bacteria able to use 2,6-DNT as the sole source of carbon, nitrogen, and energy and the initial steps in the 2,6-DNT degradative pathway.


MATERIALS AND METHODS

Isolation and growth of bacteria. Soil and groundwater samples were obtained from a number of sites contaminated by DNT (10 sites), and activated sludge samples were obtained from industrial waste treatment systems (9 sites) that receive DNT-containing waste streams. One milliliter of water or activated sludge or 1 g of soil was inoculated into 100 ml of nitrogen-free minimal medium (3) (BLK) containing 2,4-DNT (100 µM) or 2,6-DNT (50 µM) as the sole source of carbon and nitrogen. Cultures were incubated at 30°C with shaking (250 rpm). DNT concentrations were monitored by high-performance liquid chromatography (HPLC) (see below). Transfers to fresh BLK were made when concentrations of DNT in the culture fluid decreased. After several transfers (2 to 14 months), samples were spread on dilute (one-fourth strength) tryptic soy agar or on DNT plates (see below) and incubated for 1 to 6 weeks. Freshly grown isolates were inoculated into 96-well microtiter plates containing BLK (100 µM) with either 2,4-DNT (100 µM), 2,6-DNT (50 µM), or a mixture of 2,4- and 2,6-DNT (100 and 50 µM, respectively) and incubated at 30°C. After 3 to 5 days, nitrite and ammonia were measured in the culture fluids.

Strains were characterized by standard procedures (24) and by G+C and G–C microplates (Biolog, Inc., Hayward, Calif.). 16S rRNA ribosomal DNA (rDNA) analysis was provided by Fred Rainey of the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH.

Amberlite XAD-7 resin was added to some cultures to provide a gradual but continuous release of DNT. DNT was added to an empty flask to give an amount equal to a final concentration of 1 to 4 mM. The DNT was dissolved in a small amount of acetone which was evaporated under a stream of air to leave a coat of fine DNT crystals in the bottom of the flask. Appropriate amounts of BLK and XAD-7 resin (washed three times with methanol, 10 g [hydrated weight]/liter or 3.5 g [dry weight]/liter) were added to the DNT-coated flask prior to autoclaving. The procedure resulted in a final dissolved DNT concentration of 20 to 200 µM
HPLC analyses for DNT and methylnitrocatechols were performed as previously described (21) or on two Zorbax Relain CN cartridge columns connected in series (i.d. by 8 cm; 5 mm). The mobile phase consisted of a 75:25 ratio of part A (13.3 mM trifluoroacetic acid in water) to part B (6.75 mM trifluoroacetic acid in acetonitrile), delivered at a flow rate of 2 ml/min. Semipreparative HPLC was performed with an Adsorbosphere C18 column (10 mm [i.d. by 25 cm; 10 mm), with a mobile phase of 65:15 part A-part B, respectively, delivered at a flow rate of 4 ml/min. The ring cleavage products and subsequent metabolites were analyzed using a Synchropak SCD (4.6 mm[i.d.] by 25 cm; Micro Scientific, Northbrook, Ill.) reversed-phase column with a mobile phase of potassium phosphate buffer (100 mM, pH 7.0) at a flow rate of 0.5 ml/min.

Spectrophotometric analyses were performed on a Cary 3E UV-visible light (UV-VIS) spectrophotometer (Varian Associates, Sunnyvale, Calif.). Gas chromatography-mass spectrometry (GC-MS) analyses were performed on an HP5890 gas chromatograph equipped with a 30-m DB-5 fused-silica capillary column and an HP5971 mass selective detector. Liquid chromatography (LC)-MS-MS analyses were conducted by J. V. Johnson of the Department of Chemistry, University of Florida. NMR analyses were performed by T. Godrie of the NMR Laboratory, Chemistry Department, Florida State University.

RESULTS

Isolation and identification of 2,4- and 2,6-DNT-degrading bacteria. DNT disappearance accompanied by accumulation of nitrite in enrichment cultures began after several days to several weeks of incubation with 2,4-DNT provided as the sole source of carbon, nitrogen, and energy and after several weeks to several months of incubation with 2,6-DNT. Most isolated strains degraded soil and groundwater samples yielded both 2,4- and 2,6-DNT-degrading strains. Enrichments prepared using activated sludge as initial inoculum were not as successful. Many of the activated sludges transformed 2,4- and 2,6-DNT to 2-amino-4-nitrotoluene or 2-amino-6-nitrotoluene, probably through the action of nonspecific nitroreductases. The amionitrotoluenes often accumulated without further transformation, and efforts to isolate DNT-degrading strains from such cultures were discontinued. Pure cultures isolated from other enrichment cultures were tested for release of ammonia and nitrite from DNT. None of the strains released ammonia when provided with DNT as the sole carbon and nitrogen source. Strains that released substantial amounts of nitrite were transferred to fresh medium. Strains that removed DNT from culture fluids during a 7-day incubation, accompanied by stoichiometric release of nitrite and without the accumulation of aminonitrotoluenes, were considered presumptive DNT-degrading bacteria.

Approximately 30 strains that degraded 2,4-DNT were isolated from soil and surface water collected at Radford Army Ammunition Plant (Table 1). Two of the 30 strains grew notably faster on 2,4-DNT than did the only previously described 2,4-DNT-degrading isolate, Burkholderia sp. strain DNT (28). The two new isolates were determined to be B. cepacia by partial 16S rDNA analysis and designated strains R34 and PR7. Strain R34 accumulated substantially less 4M5NC during induction than did Burkholderia sp. strain DNT. Samples collected from Volunteer Army Ammunition Plant yielded three different types of 2,4-DNT-degrading strains. Two clusters that appeared distinct by Biolog GN microplate reactions gave identical partial 16S rDNA sequences and have been designated Alcaligenes sp. They are represented by strain JS867 (Alcaligenes denitrificans Biolog cluster) and strain JS871 (Alcaligenes xylosoxidans Biolog cluster). The third cluster, represented by B. cepacia strain JS872 (21), exhibited particularly rapid growth on 2,4-DNT. Depletion of 2,4-DNT (1 mM) by JS872 took place in 24 h compared to 3 to 5 days for strain DNT and 2 to 3 days for strains PR7 and R34. All 2,4-DNT-degrading strains examined to date use the same pathway as Burkholderia sp. strain DNT for degradation of 2,4-DNT. None of the 2,4-DNT-degrading isolates was capable of growth on 2,6-DNT.

Phenylthiocarbamyl derivatives of nitrogen and oxygen metabolites were measured by standard methods (24). Protein was measured as previously described (25). HPLC analyses for DNT and methylnitrocatechols were performed as previously described (21) or on two Zorbax Relain CN cartridge columns connected in series (i.d. by 8 cm; 5 mm). The mobile phase consisted of a 75:25 ratio of part A (13.3 mM trifluoroacetic acid in water) to part B (6.75 mM trifluoroacetic acid in acetonitrile), delivered at a flow rate of 2 ml/min. Semipreparative HPLC was performed with an Adsorbosphere C18 column (10 mm [i.d. by 25 cm; 10 mm), with a mobile phase of 65:15 part A-part B, respectively, delivered at a flow rate of 4 ml/min. The ring cleavage products and subsequent metabolites were analyzed using a Synchropak SCD (4.6 mm[i.d.] by 25 cm; Micro Scientific, Northbrook, Ill.) reversed-phase column with a mobile phase of potassium phosphate buffer (100 mM, pH 7.0) at a flow rate of 0.5 ml/min.

Spectrophotometric analyses were performed on a Cary 3E UV-visible light (UV-VIS) spectrophotometer (Varian Associates, Sunnyvale, Calif.). Gas chromatography-mass spectrometry (GC-MS) analyses were performed on an HP5890 gas chromatograph equipped with a 30-m DB-5 fused-silica capillary column and an HP5971 mass selective detector. Liquid chromatography (LC)-MS-MS analyses were conducted by J. V. Johnson of the Department of Chemistry, University of Florida. NMR analyses were performed by T. Godrie of the NMR Laboratory, Chemistry Department, Florida State University.
TABLE 1. Identification of selected DNT-degrading strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>Identification</th>
<th>Source*</th>
<th>DNT isomer</th>
<th>Gram stain</th>
<th>Activity</th>
<th>Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>R34</td>
<td>Burkholderia cepacia</td>
<td>Pseudomonas sp.</td>
<td>1; 1992</td>
<td>2,4</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>PR7</td>
<td>Burkholderia cepacia</td>
<td>Pseudomonas sp.</td>
<td>1; 1992</td>
<td>2,4</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>JS850</td>
<td>Burkholderia cepacia</td>
<td>None</td>
<td>2; 1995</td>
<td>2,6</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>JS863</td>
<td>Hydrogenophaga palleroni</td>
<td>None</td>
<td>3; 1995</td>
<td>2,6</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>JS867</td>
<td>Alcaligenes sp.</td>
<td>Alcaligenes denitrificans</td>
<td>2; 1995</td>
<td>2,4</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>JS871</td>
<td>Alcaligenes sp.</td>
<td>Alcaligenes xylosoxidans</td>
<td>2; 1995</td>
<td>2,4</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>JS872</td>
<td>Burkholderia cepacia</td>
<td>Pseudomonas cepacia</td>
<td>2; 1995</td>
<td>2,4</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>JS881</td>
<td>Pseudomonas putida</td>
<td>Pseudomonas sp.</td>
<td>1; 1992</td>
<td>2,6</td>
<td>−</td>
<td>−</td>
</tr>
</tbody>
</table>

* Source: 1, Radford Army Ammunition Plant, soil and surface water; 2, Volunteer Army Ammunition Plant, soil and surface water; 3, West Virginia, activated sludge.

The first 2,6-DNT-degrading strains (Table 1) were enriched from soil samples from the Volunteer Army Ammunition Plant. The partial 16S rDNA sequence of the isolate that grew most rapidly on 2,6-DNT, strain JS863, was 99% identical to the 16S rDNA of Hydrogenophaga palleroni. B. cepacia strain JS850, also identified by partial 16S rDNA analysis, was isolated from activated sludge from an industrial waste treatment system of a DNT-manufacturing facility. None of the 2,6-DNT-degrading isolates could grow with 2,4-DNT provided as the sole growth substrate.

Inhibition of 2,4-DNT degradation by 2,6-DNT. 2,4-DNT-grown cultures of strains DNT, PR7, and R34 were provided with mixtures of 2,4- and 2,6-DNT. After 3 days (Fig. 1), 2,4-DNT remained in all cultures provided with 200 μM or more 2,6-DNT while cultures without added 2,6-DNT completely removed 2,4-DNT within 1 to 2 days. When the strains were grown on succinate with NH₄Cl added as nitrogen source, addition of 2,4- or 2,6-DNT at concentrations of ≥100 μM reduced the cell densities achieved during a 24-h incubation, whereas 10 and 25 μM DNT had little effect on the final cell densities. The results indicate that high concentrations of either isomer of DNT inhibit growth of DNT-degrading strains on simple substrates.

Production and identification of 3M4NC. HPLC analysis revealed the accumulation of a yellow metabolite in culture fluids after growth of strains PR7 and R34, but not in culture fluids of strain DNT, when 2,6-DNT was present with 2,4-DNT. The metabolite had a different HPLC retention time from that of 4M5NC, but the pH-dependent UV-VIS spectrum...
and 2,4-DNT was not markedly inhibited (Table 3). The results suggested that the inability of 2,4-DNT-grown cells to metabolize 2,4-DNT in the presence of 2,6-DNT was not due to the effect of 2,6-DNT on the 2,4-DNT dioxygenase or the 4M5NC monooxygenase. In addition, neither 3M4NC nor 4M5NC, each of which is highly stable at room temperature under aqueous conditions, has ever been detected in DNT-contaminated soil. We conclude that, if a metabolite is involved in inhibition of DNT degradation, it is not one of the methylnitrocatechols.

Growth of 2,6-DNT-degrading bacteria. Growth on 2,6-DNT was inhibited in the presence of other carbon sources tested (glucose, succinate, acetate, glycerol, yeast extract, aspartate, glutamate, alanine, and nitrite). Growth on 2,6-DNT was completely inhibited. When 2,6-DNT (1 to 3 mM) was provided as the sole source of carbon and energy, 2,6-DNT degradation was inhibited by being adsorbed to XAD-7 resin, both nitrite and protein in the presence of other carbon sources tested (glucose, succinate, acetate, glycerol, yeast extract, aspartate, glutamate, alanine, and nitrite). Growth on 2,6-DNT was inhibited in the presence of other carbon sources tested (glucose, succinate, acetate, glycerol, yeast extract, aspartate, glutamate, alanine, and nitrite).

Toxicity of 3M4NC to 2,4-DNT-degrading bacteria. 4M5NC was highly toxic to strain DNT at concentrations above 2 μM (9). Accumulation of 3M4NC in culture fluids of 2,4-DNT-grown strains provided with 2,6-DNT suggested that 3M4NC might be toxic to 2,4-DNT-degrading strains. The purified 4M5NC monooxygenase from strain DNT (9) was assayed for its ability to oxidize 3M4NC. Concentrations of 1 to 10 μM 3M4NC were not toxic to strain DNT at concentrations above 2 μM (9). Accumulation of 3M4NC in culture fluids of 2,4-DNT-grown strains provided with 2,6-DNT suggested that 3M4NC might be toxic to 2,4-DNT-degrading strains. The purified 4M5NC monooxygenase from strain DNT (9) was assayed for its ability to oxidize 3M4NC. Concentrations of 1 to 10 μM 3M4NC were not toxic to strain DNT at concentrations above 2 μM (9).

Accumulation of 3M4NC in culture fluids of 2,4-DNT-grown DNT PR7 JS867 JS871 was not markedly inhibited (Table 3). The results suggest that the inability of 2,4-DNT-grown cells to metabolize 2,4-DNT in the presence of 2,6-DNT was not due to the effect of 2,6-DNT or 3M4NC on the 2,4-DNT dioxygenase or the 4M5NC monooxygenase. In addition, neither 3M4NC nor 4M5NC, each of which is very stable at room temperature under aqueous conditions, has ever been detected in DNT-contaminated soil. We conclude that, if a metabolite is involved in inhibition of DNT degradation, it is not one of the methylnitrocatechols.
TABLE 4. Oxygen consumption by washed cells after growth on 2,6-DNT

<table>
<thead>
<tr>
<th>Substrate</th>
<th>O₂ uptake (nmol/min/mg of protein) by strain:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>JS850</td>
</tr>
<tr>
<td>2,6-DNT</td>
<td>7.5</td>
</tr>
<tr>
<td>3M4NC</td>
<td>11.5</td>
</tr>
<tr>
<td>2,3,6-Trihydroxytoluene</td>
<td>14.4</td>
</tr>
<tr>
<td>4M5NC</td>
<td>0.6</td>
</tr>
<tr>
<td>3-Methyl-6-nitrocatechol</td>
<td>0.2</td>
</tr>
<tr>
<td>4-Nitrocatechol</td>
<td>1.0</td>
</tr>
<tr>
<td>Catechol</td>
<td>5.5</td>
</tr>
<tr>
<td>Toluene</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>TNT</td>
<td>1.6</td>
</tr>
<tr>
<td>2,4-DNT</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>2,6-Dinitrophenol</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>2-Methyl-3-nitrophenol</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>2-Amino-6-nitrotoluene</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>2,4,5-Trihydroxytoluene</td>
<td>0.3</td>
</tr>
</tbody>
</table>

* Substrates were provided at 50 μM.

- Yeast extract- and tryptic soy broth-grown cells showed no increased uptake with 2,6-DNT and 3M4NC. Other substrates were not tested.
- Number in parentheses is stoichiometry of O₂ consumption.

DNT-degrading strains have a narrow substrate specificity. Lack of increased oxygen uptake after incubation with 2-methyl-3-nitrophenol and 2-amino-6-nitrotoluene also suggested that neither a monooxygenase nor a nitroreductase was involved in the initial attack.

Enzyme studies. Crude cell extracts prepared from 2,6-DNT-grown strains JS863 and JS850 converted 3M4NC to a yellow compound (compound Y) that only slowly disappeared upon overnight incubation. Typical reaction rates for conversion of 3M4NC to compound Y were 35 ± 6 nmol/min/mg of protein. Compound Y had an absorbance maximum at 375 nm. The A₃75 decreased upon acidification and returned when the mixture was returned to a neutral pH or made basic. The behavior of compound Y was consistent with that of a meta-ring cleavage product. Repetitive scans during the reaction revealed an isosbestic point at 435 nm (Fig. 5).

Addition of 3M4NC to compound Y by crude cell extracts of JS863. An extract from a 2,6-DNT-grown culture was incubated in phosphate buffer (20 mM, pH 7.0) with 100 μM 3M4NC. The reaction was initiated by the addition of the catechol (scan 1). Scans were recorded at 2-min intervals. Compound Y had a single absorbance maximum at 375 nm.
ferrous or ferric iron, NAD+, or NADP+ did not affect the reaction. Preincubation of cell extract (100 μl) with H2O2 (5 μl of 30%) greatly reduced the enzyme activity. The activity was stable in frozen extracts but was reduced by 90% in extracts heated to 50°C for 10 min and abolished in extracts that were heated to 55°C for 15 min. No nitrite release was detected upon conversion of 3M4NC to compound Y. The enzyme was not active with 4M5NC, 3-methyl-6-nitrocatechol, 4-nitrocatechol, 2-hydroxy-3-methylquinone, or 2,3,6-trihydroxytoluene; however, 3-methylcatechol was slowly converted (at a rate 32 to 35% of that observed with 3M4NC) to a yellow compound (compound Z) with absorbance maxima at 385 and 320 nm, which are identical to those of the product of meta-ring cleavage of 3-methylcatechol by toluene-grown Pseudomonas putida (14). Neither 3M4NC nor 3-methylcatechol was transformed by cell extracts prepared from strain JS850 or JS863 grown on tryptic soy broth.

When 3M4NC was incubated with cell extracts heated to 50°C for 10 min, 3M4NC was converted to a yellow product (compound X) whose absorbance spectrum differed from that of compound Y. Compound X had absorbance maxima at 394 and 326 nm at pH 7.0. Subsequent addition of an unheated extract of JS850 or JS863 converted 3M4NC to compound X. Unheated extract of JS850 or JS863 converted 3M4NC to compound Y in solutions buffered between pH 7 and pH 10. The reaction was inhibited at pH 4 and pH 11. At pH 5, compound X was produced and only slowly transformed to compound Y. The results suggest that the crude cell extracts convert 3M4NC to compound Y via compound X in at least two enzymatic steps.

Cell extracts prepared from E. coli JM109 containing pDTG603 (carrying the cloned ioe gene encoding 3-methylcatechol-2,3-dioxygenase) converted 3M4NC to a compound with an absorbance spectrum identical to that of compound X. Addition of unheated cell extracts of JS850 or JS863 converted the compound to one with an absorbance spectrum identical to that of compound Y (Fig. 6) (initial rate, 36 ± 1 nmol/min/mg of protein). Cell extracts containing pDTG603 converted 3-methylcatechol to 2-hydroxy-6-oxohepta-2,4-dienoic acid (30), which had an absorbance spectrum and an HPLC retention time identical to those of compound Z. Addition of cell extracts of JS850 or JS863 had no effect on the spectrum of 2-hydroxy-6-oxohepta-2,4-dienoic acid. 3-Methylcatechol-2,3-dioxygenase catalyzed the complete conversion of 3-methylcatechol 15 times faster than the complete conversion of 3M4NC. The rate of conversion of 3M4NC declined rapidly after the reaction was initiated by addition of substrate. Conversion of the last 40% of the substrate required 2.5 times as much time as the conversion of the first 60% of the substrate. We did not distinguish between loss of enzyme activity and product inhibition. Extracts from JS850 and JS863 had the same rate for the entire reaction period. The reaction rates given above are consistent with oxygen uptake rates. The results indicate that the enzyme that produced compound X from 3M4NC is a catechol-2,3-dioxygenase with a different substrate specificity from that of 3-methylcatechol-2,3-dioxygenase.

Identification of compounds X and Y. Compounds X and Y were accumulated and purified as described in Materials and Methods. Compound X was unstable at low pH and did not partition into organic solvents at neutral pH, necessitating concentration of the compound by lyophilization without further purification. LC-MS of compound X revealed a base peak at m/z 200 (Fig. 3B) which corresponded to the [M—H]− ion. The fragment with a m/z of 120 is an artifact of a gas-phase reaction involving acetate-acetic acid from the LC mobile phase and, as demonstrated by LC-MS-MS of the 200 mass ion (data not shown), is unrelated to compound X. A [M—H]− mass of 200 is consistent with that expected for a dioxygenolytic product of 3M4NC. LC-MS-MS of the [M—H]− ion resulted in a fragment with a m/z of 129 ([H2C-C≡O=O]=—C(≡NO2)=CH—CH3) and MS analysis of the fragment of m/z 129 revealed fragment ion m/z 86 ([O2N—CH—CH3]−) and m/z 59 ([O2N—CH]−). The observed fragmentation is consistent with the product of proximal meta-ring cleavage of 3M4NC but not with the product of distal meta-ring cleavage or ortho-ring cleavage of 3M4NC.

The 13C NMR of compound X revealed seven carbon resonances for which putative assignments have been made, and 1H NMR showed a set of coupled protons and a singlet peak at 2.5 ppm arising from protons of a methyl group (Table 2). The results are consistent with the structure of the 3M4NC proximal meta-ring cleavage product, 2-hydroxy-5-nitro-6-oxohepta-2,4-dienoic acid.

Compound Y was stable (several minutes) under acidic conditions but decomposed upon overnight storage at pH 3.5. The LC-MS of compound Y (Fig. 3C) revealed a base peak at m/z 158 and, as further demonstrated by LC-MS-MS of the [M—H]− ion, a major fragment at m/z 86 ([O2N—CH—CH—CH3]−). As described above, the peak at m/z 120 is an artifact. The 13C and 1H NMR spectra (Table 2) revealed five carbon resonances and three olefinic proton signals that were nearly identical to those previously reported for 2-hydroxy-5-nitropenta-2,4-dienoic acid (6). The mass spectrum and NMR analyses unequivocally identified compound Y as 2-hydroxy-5-nitropenta-2,4-dienoic acid. The structure of 2-hydroxy-5-nitropenta-2,4-dienoic acid is consistent with the postulated product of the hydrolytic loss of acetate from 2-hydroxy-5-nitro-6-oxohepta-2,4-dienoic acid.

**DISCUSSION**

DNT-contaminated sites around TNT-manufacturing plants have been the source of many 2,4-DNT-degrading bacterial strains and, as reported here, 2,6-DNT-degrading bacteria. Soil...
slurry reactors (21) and fluid bed reactors (15) inoculated with mixtures of specific DNT-degrading strains have demonstrated degradation and mineralization (21) of mixtures of the 2,4- and 2,6-DNT isomers. Preliminary evidence indicates, however, that in mixed cultures, high concentrations of 2,6-DNT inhibit the degradation of 2,4-DNT and high concentrations of 2,4-DNT inhibit the degradation of 2,6-DNT. We determined the initial steps in the 2,6-DNT catabolic pathway in order to gain an understanding of how the DNT isomers affect overall DNT degradation when both 2,4- and 2,6-DNT are present. To our knowledge, this is the first description of the degradative pathway for 2,6-DNT and the first reported bacterial production of 3M4NC and 2-hydroxy-5-nitro-6-oxohepta-2,4-dienoic acid.

The transient accumulation of 3M4NC during induction of the 2,6-DNT degradation pathway suggested that 3M4NC is a pathway intermediate. Simultaneous induction studies indicated that 3M4NC is the product of an initial dioxygenation as in the 2,4-DNT degradation pathway, rather than sequential monooxygenation of 2,6-DNT to 2-methyl-3-nitrophenol and then 3M4NC. The results of the simultaneous induction studies and the release of both nitro groups of 2,6-DNT as nitrite suggested that the 2,6-DNT pathway might be analogous to the 2,4-DNT pathway (Fig. 7A). However, neither simultaneous induction nor enzyme assays indicated that 2,3,6-trihydroxytoluene, the 2,6-DNT analog of 2,4,5-trihydroxytoluene, of the 2,4-DNT pathway, was involved in the 2,6-DNT catabolic pathway. Studies with cell extracts conclusively demonstrated that, after the initial dioxygenation, the two pathways diverged.

Enzyme assays with crude and partially purified cell extracts revealed the presence of an extradiol ring cleavage dioxygenase and a hydrolase that catalyzed reactions subsequent to the initial dioxygenation of 2,6-DNT. The nitro group of 3M4NC is eliminated by unknown reactions subsequent to ring fission. In contrast, the nitro group of 4MSNC is eliminated prior to ring cleavage in the 2,4-DNT degradative pathway (8, 28). Elimination of the nitro group prior to ring fission is also the case in pathways for degradation of 4-nitrophenol (12, 27), nitrobenzene (20), 2-nitrotoluene (10), 3-nitrotoluene (1), and 3-nitrobenzoate (4, 19). Only a few other pathways have been reported in which a nitroaromatic compound is the result of ring cleavage. Rhodococcus erythropolis HL 24-1 and HL 24-2 produced the dead-end metabolite 4,6-dinitrohexanoate during growth on 2,4-dinitrophenol (17). Later studies showed the reductive production of analogous dead-end metabolites when the strains were grown on substituted 2,4-dinitrophenols (16). Rhodococcus sp. strain RB1 grew on 2,4-dinitrophenol via 3-nitrophenol (2). The authors could not eliminate the possibility that 4,6-dinitrohexanoate was produced by cleavage of a hypothetical Meisenheimer intermediate and was then oxidized to 3-nitroaniline. A. eutrophus JMP 134 grew on 2,4-dinitrophenol, with the stoichiometric release of nitrite (6). The key intermediate in the pathway is 4-nitropyrogallol, which serves as the ring fission substrate. The ring of 4-nitropyrogallol was opened between the 2- and 3-hydroxy positions to yield 2-hydroxy-5-nitromuconic acid. The steps leading to the subsequent elimination of the second nitro group have not been determined for the A. eutrophus strains. 2-Hydroxy-5-nitropenta-2,4-dienoic acid is thought to be a dead-end product of spontaneous decarboxylation of the nitromuconic acid. In contrast to the above pathways, 2-hydroxy-5-nitropenta-2,4-dienoic acid synthesis is enzyme catalyzed in the 2,6-DNT pathway. The fact that 2-hydroxy-5-nitropenta-2,4-dienoic acid has not been detected in the culture fluids during growth on 2,6-DNT suggests that it is subject to further productive metabolism by 2,6-DNT-degrading bacteria.

Catechol undergoes meta-cleavage to 2-hydroxy-6-oxohepta-2,4-dienoic acid (hydroxymuconic semialdehyde), which can be converted to 2-oxopent-4-enoic acid in two ways (18). An NAD+ -dependent dehydrogenase can oxidize the hydroxymuconic semialdehyde to the enol form of 4-oxalocrotonate which is converted to the keto form by the action of a tautomerase. The keto compound is enzymatically decarboxylated to 2-oxopent-4-enoic acid. Alternatively, the hydroxymuconic semialdehyde may be directly converted to 2-oxopent-4-enoic acid by the action of a hydrolase. In contrast, in the 3-methylcatechol and the 2,6-DNT degradative pathways, the hydroxymuconic semialdehyde analogs have methyl group substituents on the 6-carbon so that the oxo groups exist as ketones rather than aldehydes and cannot be acted upon by a dehydrogenase. Thus, direct enzymatic hydrolysis of 2-hydroxy-4-nitro-6-oxohepta-2,4-dienoic acid with loss of acetate is the only plausible route to 2-hydroxy-5-nitropenta-2,4-dienoic acid. The additional observation that cell extracts converted 3M4NC only to 2-hydroxy-5-nitro-6-oxohepta-2,4-dienoic acid at pH 5.0 is consistent with inhibition of a hydrolase. The aromatic ring cleavage product hydrolases are serine hydrolases, members of the aβ hydrolase fold family (5). Enzymes of this family require the deprotonation of a serine to generate a nucleophilic resi-
due in the active site (22). At acidic pH, the active-site serine would remain protonated, reducing the catalytic efficiency of the hydrolase.

Crude cell extracts from induced cultures of JS850 and JS863 eliminate the nitro group from nitroalkanes (nitroethane and nitromethane) without any additional cofactors (data not presented), but the activity remains to be linked to the release of nitrite from a nitroaliphatic intermediate in the 2,6-DNT catabolic pathway. If the activity is indeed part of the 2,6-DNT pathway, then nitrite release is at least two enzyme reactions down the pathway from 2-hydroxy-5-nitrophenol to 2,4-dienoic acid production. That is, transformation of 2-hydroxy-5-nitrophenol into 2,4-dienoic acid which has not been detected in cell extracts would require at least one reaction to yield a product that the nitro group-eliminating activity could then act upon.

Based on the above results, we propose the following pathway for 2,6-DNT degradation (Fig. 7B). Dioxygenase attack at either of the nitro groups converts 2,6-DNT to 3M4NC with the elimination of nitrite. The aromatic ring is opened by an extradiol ring cleavage dioxygenase resulting in 2-hydroxy-5-nitro-6-oxohexa-2,4-dienoic acid. By analogy to the 3-methylcatechol meta-ring cleavage pathway, hydrolytic attack would produce 2-hydroxy-5-nitrophenol to 2,4-dienoic acid accompanied by the loss of acetate.

Previous work demonstrated that the highly specific 4M5NC monoxygenase from the 2,4-DNT pathway does not attack 3M4NC (9). The 3M4NC-2,3-dioxygenase from the 2,6-DNT pathway also appears to be highly specific for 3M4NC, with the only other substantial activity being against 3-methylcatechol. Because there was no detectable activity with 4-nitrocatechol, it seems that the methyl group in the 3-position is the determining factor in substrate recognition by the enzyme. In addition, 3-methyl-6-nitrocatechol was not attacked, which suggests that compounds that are substituted at the 6-position are not recognized by the enzyme.

3M4NC was initially identified in 2,4-DNT-degrading cultures that were incubated with 2,6-DNT. This result was not surprising, as we had previously reported the limited ability of the 2,4-DNT-degrading strain DNT to transform other nitrocompounds (28). Additionally, the genes that encode the α subunit of the nitroarene dioxygenases have been shown to share a striking degree of nucleotide sequence similarity (G. R. Johnson and J. C. Spain, Abstr. 97th Gen. Meet. Am. Soc. Microbiol., p. Q-344, p. 512, 1997). We were able to use a cloned 2,4-DNT dioxygenase gene to synthesize substantial quantities of 3M4NC from 2,6-DNT, but 2,4-DNT was clearly the preferred substrate. Although the cloned genes were overexpressed, the yields were low, generally around 30%, perhaps indicating that only subtle changes in amino acid sequence are required to affect substrate specificity or perhaps indicating that some crucial component of the regulatory system was missing in the clone.

The above observation and the puzzling question of why DNT persists in environments known to harbor effective DNT-degrading organisms highlight our lack of understanding of the induction and regulation of the DNT degradative pathways. A related question concerns the evolutionary origin and distribution of the genes involved in DNT degradation. The genes for the initial dioxygenases involved in 2,4- and 2,6-DNT degradation are all closely related, even though the distribution of the organisms harboring the genes is quite discontinuous. And while the initial dioxygenases are closely related, the remainder of the pathways are clearly different. The difference raises the question of why divergent pathways evolved to degrade the two isomers. We are currently working to determine the mechanisms involved in regulating the degradation of mixtures of DNT.

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