

Biodegradation of 2,4-Dinitrotoluene by a *Pseudomonas* sp.

RONALD J. SPANGGORD,¹ JIM C. SPAIN,^{2*} SHIRLEY F. NISHINO,² AND KRISTIEN E. MORTELMANS¹

SRI International, Menlo Park, California 94025,¹ and Air Force Engineering and Services Center, Tyndall Air Force Base, Florida 32403-6001²

Received 23 April 1991/Accepted 21 August 1991

Previous studies of the biodegradation of nonpolar nitroaromatic compounds have suggested that microorganisms can reduce the nitro groups but cannot cleave the aromatic ring. We report here the initial steps in a pathway for complete biodegradation of 2,4-dinitrotoluene (DNT) by a *Pseudomonas* sp. isolated from a four-member consortium enriched with DNT. The *Pseudomonas* sp. degraded DNT as the sole source of carbon and energy under aerobic conditions with stoichiometric release of nitrite. During induction of the enzymes required for growth on DNT, 4-methyl-5-nitrocatechol (MNC) accumulated transiently in the culture fluid when cells grown on acetate were transferred to medium containing DNT as the sole carbon and energy source. Conversion of DNT to MNC in the presence of ¹⁸O₂ revealed the simultaneous incorporation of two atoms of molecular oxygen, which demonstrated that the reaction was catalyzed by a dioxygenase. Fully induced cells degraded MNC rapidly with stoichiometric release of nitrite. The results indicate an initial dioxygenase attack at the 4,5 position of DNT with the concomitant release of nitrite. Subsequent reactions lead to complete biodegradation and removal of the second nitro group as nitrite.

2,4-Dinitrotoluene (DNT) is the major impurity resulting from the manufacture of 2,4,6-trinitrotoluene (TNT) and is a primary starting material for the synthesis of toluenediisocyanate, which is used in the production of polyurethane foam. Wastes from these processes have contaminated waterways and soils, and because of its toxicity, DNT is listed as a priority pollutant by the U.S. Environmental Protection Agency (14).

The biodegradation of polar nitroaromatic compounds by bacteria has been studied in a number of isolates. *p*-Nitrophenol (25) and *o*-nitrophenol (31) undergo an initial attack by a monooxygenase which results in the release of nitrite and the formation of the corresponding dihydroxy compound. In contrast, *m*-nitrophenol is first reduced to the corresponding aminophenol, which is further degraded by an unknown mechanism (31). 3,5-Dinitro-2-methylphenol (dinitro-*o*-cresol) can be degraded via either the oxidative (12) or the reductive route (29). Nitrobenzoates appear to be degraded primarily by the oxidative pathway (5, 6).

In contrast to the nitrophenols and nitrobenzoates, nonpolar nitroaromatic compounds such as nitrobenzene and the nitrotoluenes are more resistant to microbial attack. Early work suggested that reduction was the primary reaction mediated by microorganisms. Thus, McCormick et al. (17, 18) identified azoxy compounds and amino derivatives from the biotransformation of di- and trinitrotoluenes and Liu et al. (16) identified amino and nitroso compounds from anaerobic biotransformation of DNT. Similarly, nitrobenzenes and nitrotoluenes were reduced in sewage (13); however, none of the reduction products seemed to be further degraded, and in many instances they were more toxic than the parent nitro compounds. Dey et al. (8) also reported isolation of 16 bacterial and fungal strains able to degrade *m*-dinitrobenzene.

Recent work from several laboratories suggests that nitrotoluenes can be biodegraded by an oxidative pathway. *Phanerochaete chrysosporium* completely mineralizes TNT by an unknown mechanism under aerobic conditions (10).

Unkefer et al. (30) have isolated a *Pseudomonas* sp. that mineralizes TNT with concomitant release of nitrite. Several isolates able to degrade TNT by a reductive pathway were reported by Neumeier et al. (19). Kulpa et al. (15) have studied the aerobic degradation of TNT by a mixed culture.

In spite of the preliminary evidence that nitrotoluenes can be degraded by an oxidative pathway, to our knowledge there is no information on the initial steps in the process. We report here the isolation and characterization of a *Pseudomonas* strain able to use DNT as the sole source of carbon. We also present evidence based on identification of an early metabolite that the initial attack catalyzed by a dioxygenase involves displacement of the 4-nitro group by molecular oxygen.

MATERIALS AND METHODS

Isolation and growth of bacteria. Water samples from Waconda Bay, located near the Volunteer Army Ammunition Plant in Chattanooga, Tenn., were supplemented with DNT (100 mg/liter) and incubated for 5 days at 25°C. Particulate material was collected by centrifugation and transferred to a minimal medium containing the following (in grams per liter): DNT, 0.1; K₂HPO₄, 0.7; KH₂PO₄, 0.3; (NH₄)₂SO₄, 0.5; NaCl, 0.5; MgSO₄ · 7H₂O, 0.05; CaCl₂ · 2H₂O, 0.1; FeSO₄ · 7H₂O, 0.003; and 1.0 ml of trace elements solution per liter. The trace elements solution contained the following (per liter): 0.1 g of H₃BO₃ and 0.05 g (each) of CaSO₄ · 5H₂O, ZnSO₄ · 7H₂O, and Na₂MoO₄ · 6H₂O. Cultures were incubated for 5 days with shaking, and then samples were spread on agar plates supplemented with yeast extract (YE; 2 g/liter) for isolation of individual colonies.

Strains were characterized by using standard procedures (22) and with Biolog GN Microplates (Biolog, Inc., Hayward, Calif.). Auxanography (20) was used to screen DNT-degrading strains for ability to grow on other nitroaromatic compounds.

Cultures were maintained on a minimal salts medium (MSB) (24) containing DNT (100 mg/liter) and YE (200 mg/liter). For experiments with induced cells, cultures were

* Corresponding author.

grown overnight in 1.5 liters of MSB supplemented with YE (200 mg/liter), acetate (0.02 M), or succinate (0.02 M) at 30°C with shaking (200 rpm). Cells were harvested by centrifugation and washed twice with fresh MSB before use in subsequent experiments.

$^{18}\text{O}_2$ incorporation. $^{18}\text{O}_2$ incorporation experiments were conducted as described previously (26) with the following modifications. Cells grown overnight on DNT and acetate (0.02 M) were suspended to a final density of 0.36 A_{600} unit (0.086 mg of protein per ml) in 500 ml of MSB. The suspensions were allowed to equilibrate with $^{18}\text{O}_2$, and DNT (500 mg/ml in *N,N*-dimethylformamide) was added to a final concentration of 5.5×10^{-4} M. An identical control culture was incubated in air and monitored by high-performance liquid chromatography (HPLC; see below) for DNT disappearance and metabolite appearance. After 2.5 h, cells were removed from the culture by centrifugation and metabolic products were extracted from the supernatant and analyzed by HPLC and gas chromatography-mass spectrometry (GC-MS).

Isolation of metabolites. Cells were grown overnight with stirring and aeration at 30°C in 8 liters of MSB containing succinate (2 g/liter). Cells were harvested and washed by filtration with a Pellicon Cassette System (Millipore Corp., Bedford, Mass.) equipped with a 0.45- μm -pore-size Duro-pore cassette. Cells were suspended in 12 liters of MSB supplemented with DNT (100 mg/liter) and incubated at 30°C with aeration and stirring for 10 h, and then an additional 500 mg of DNT was added to the culture. The cells were removed by filtration after a total of 26 h of incubation. The culture fluid was extracted with ethyl acetate, the extract was dried over anhydrous sodium sulfate, and the ethyl acetate was removed by flash evaporation at 30°C.

The primary metabolite was removed from the residue by repeated washings with small volumes of water and then extracted from the water with ethyl acetate. The solvent was removed by flash evaporation, and the dried residue was taken up in acetonitrile-water (3:7) and purified by semi-preparative HPLC.

Analytical methods. Protein was measured by the bicinchoninic acid method (23). Nitrite release was measured as described by Smibert and Krieg (22).

HPLC analyses were performed on a Spherisorb C8 column (Alltech, Deerfield, Ill.) with acetonitrile and trifluoroacetic acid (13.5 mM) as the mobile phase. The elution gradient was 25% acetonitrile and 75% aqueous trifluoroacetic acid changed to 50% acetonitrile and 50% aqueous trifluoroacetic acid over 15 min with a flow rate of 1.5 ml/min. All compounds were detected by UV A_{230} with an HP1040A diode array detector (Hewlett-Packard Corp., Palo Alto, Calif.). Semipreparative HPLC was performed on a Zorbax octyldecyl silane column (9.4 by 250 mm; DuPont Co., Wilmington, Del.). The gradient consisted of 40% acetonitrile and 60% aqueous trifluoroacetic acid changed to 50% acetonitrile and 50% aqueous trifluoroacetic acid over 10 min with a flow rate of 3.5 ml/min.

Hydroxylated compounds were derivatized with bistrifluoromethylsilylacetylamide (Pierce Chemical Co., Rockford, Ill.) and identified by GC-MS with a Ribermag R-10-10 or an HP5890 GC-MS system and a 30-m DB-5 fused silica capillary column.

Liquid chromatography-MS was performed with a Spherisorb C8 column linked to an HP5987 mass spectrometer (Hewlett-Packard) with an HP59980A particle beam interface. The mobile phase was acetonitrile-water (1:1) with a

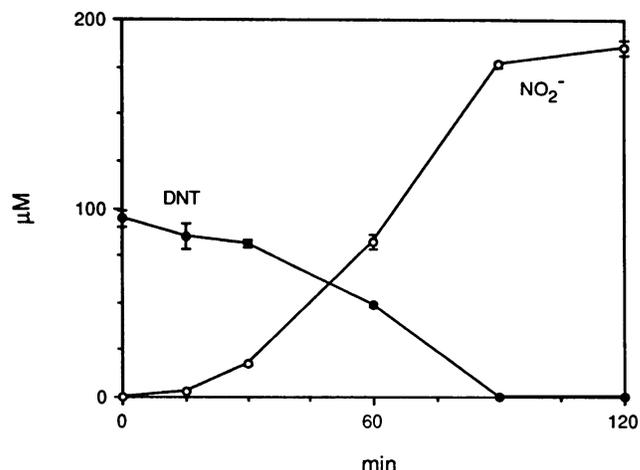


FIG. 1. Disappearance of DNT and release of nitrite into the culture medium by DNT-grown cells. Points plotted are means of triplicate cultures; bars represent 1 standard deviation.

flow rate of 0.5 ml/min; detection was by electron impact ionization.

Nuclear magnetic resonance was performed with a Varian 400 MHz spectrometer (Sunnyvale, Calif.) on solutions prepared in D_2O .

Chemicals. DNT was from Pfaltz and Bauer (Stamford, Conn.) or Aldrich Chemicals (Milwaukee, Wis.). 2,3-Dinitrotoluene, 2,6-dinitrotoluene, 3,4-dinitrotoluene, catechol, 4-nitrocatechol, 3-methylcatechol, and 4-methylcatechol were also from Aldrich Chemicals. 4-Methyl-5-nitrocatechol (MNC) was synthesized by the method of Cousin (7). 3-Nitrocatechol was prepared by the method of Astle and Stephenson (1). Catechols other than MNC were purified by vacuum sublimation. All other chemicals were of the highest purity commercially available.

RESULTS

Isolation of organisms and growth on DNT. A mixed culture from Waconda Bay degraded DNT after an incubation period of 5 days. When the medium contained [^{14}C -ring]DNT, 59% of the radioactivity was released as $^{14}\text{CO}_2$ (28). Four pure cultures were isolated from the mixture. One of the strains degraded DNT as the sole source of carbon and energy. The strain was an oxidase- and catalase-positive, motile, gram-negative rod. On the basis of these characteristics and the Biolog GN array the strain was identified as *Pseudomonas* sp. strain DNT. *Pseudomonas* sp. strain DNT grew on DNT at concentrations approaching the solubility of DNT in water (187 mg/liter), and induced cells were not inhibited when an excess of DNT crystals was present in the medium. Degradation of DNT was accompanied by the release of 2 mol of nitrite per mol of DNT (Fig. 1). DNT was not transformed in the absence of oxygen. These initial results indicated that DNT was mineralized by an oxidative pathway and that the nitro groups were removed without prior reduction to amines.

Metabolite accumulation. When washed suspensions of DNT-grown cells were transferred to fresh media containing DNT as the sole carbon source, the DNT disappeared rapidly with little or no accumulation of intermediate metabolites. When succinate- or acetate-grown cells were transferred to media containing DNT a lag period was followed by

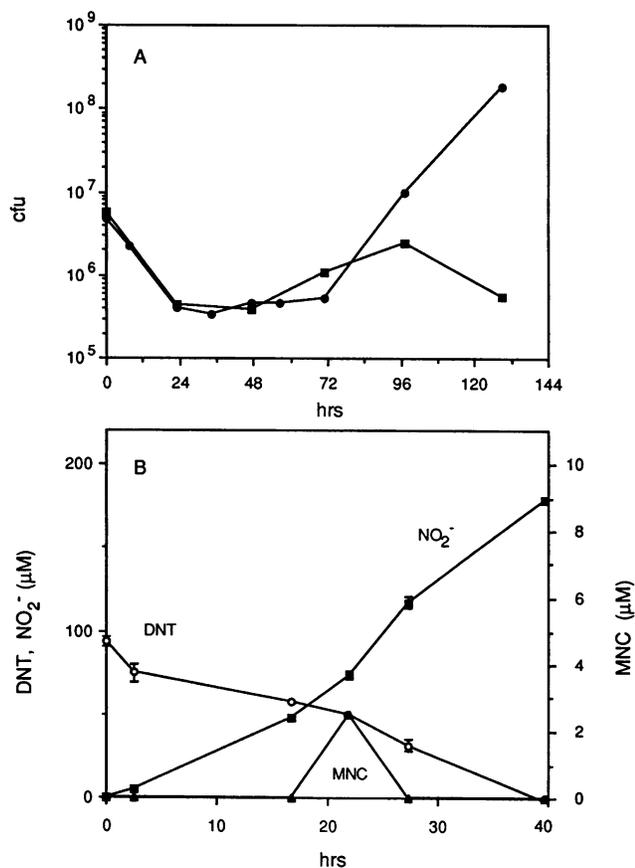


FIG. 2. Growth on DNT. Acetate-grown cells were transferred to MSB containing DNT or into control flasks without DNT. (A) CFU from DNT (500 μM) flasks (\bullet) and control flasks (\blacksquare) were determined by plate counts on DNT agar. (B) Disappearance of DNT (100 μM) and release of NO_2^- and a yellow metabolite (MNC) were measured in a separate experiment. Points plotted are averages of duplicate cultures; bars are 1 standard deviation.

the transient appearance of a more polar, yellow metabolite and nitrite release was delayed (Fig. 2). CFU increased only in cultures with DNT. Cultures differing only in the concentration of DNT added showed proportionally greater increases in CFU at the time of complete DNT removal (data not shown). Differences between numbers for tryptic soy agar and DNT agar plates were not significant.

The above-described experiment was repeated on a larger scale, and the yellow metabolite was extracted and purified. The product (350 mg) isolated from batch culture was judged to be pure by HPLC. Analysis by liquid chromatography-MS revealed an apparent molecular ion of 169 and a base peak of 152, which is typical of the loss of a hydroxyl group expected in the case of an aromatic nitro group ortho to a methyl group (4) (Fig. 3). The trimethylsilyl derivative, analyzed by GC-MS, gave an apparent molecular ion of 313, a base peak of 73 because of the trimethylsilyl group, and a major fragment at 296 (loss of the hydroxyl group [Fig. 4A]). Nuclear magnetic resonance showed resonances at 2.28, 6.57, and 7.38 ppm and area ratios of 3:1:1 corresponding to the methyl and aromatic proton groups, respectively. The mass, nuclear magnetic resonance, UV spectrum, melting point, and HPLC retention times were identical to those of authentic 4-methyl-5-nitrocatechol.

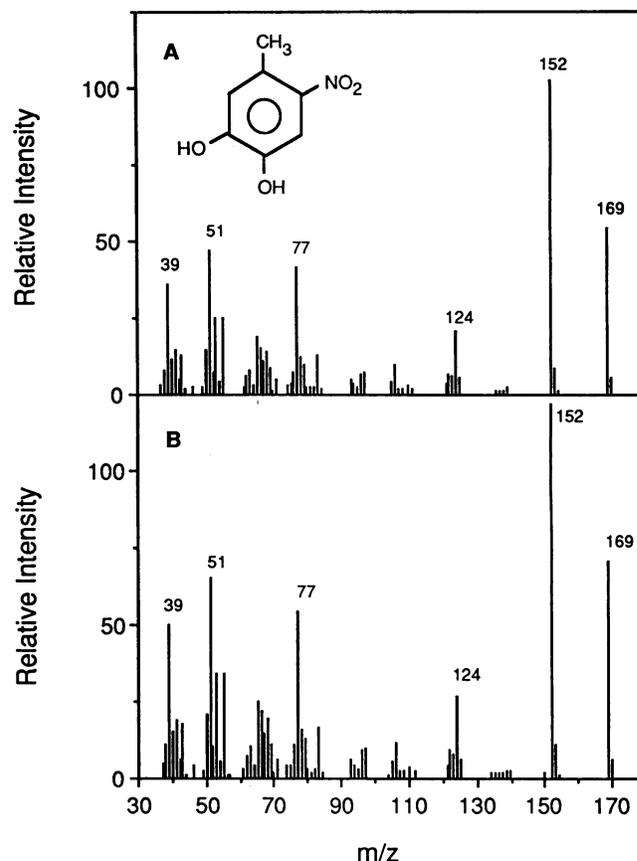


FIG. 3. Mass spectra of authentic MNC (A) and yellow metabolite from culture fluid (B).

Incorporation of ¹⁸O₂. Molecular oxygen can be incorporated into the aromatic ring by the action of monooxygenase or dioxygenase enzymes. When cells were allowed to oxidize DNT in the presence of an atmosphere containing 66.3% ¹⁶O₂ and 33.7% ¹⁸O₂, the resulting MNC showed no evidence of incorporation of a single atom of molecular oxygen. GC-MS analysis of the trimethylsilyl derivative revealed that 66.8% of the molecules contained two atoms of ¹⁶O₂ and 33.2% contained two atoms of ¹⁸O₂ (Fig. 4).

Metabolism of MNC. When the extracted and purified MNC was incubated with suspensions of DNT-grown cells (0.3 A₆₀₀ unit), it was rapidly degraded with the release of 1 mol of nitrite (Fig. 5). Identical results were obtained with the synthetic MNC.

Respirometry. Washed suspensions of DNT-grown cells rapidly oxidized DNT, MNC, and 1,2,4-benzenetriol (Table 1). Lower rates of oxygen consumption were seen when 2-nitrotoluene, 4-nitrocatechol, and 3-methyl-4-nitrophenol were provided as substrates. Acetate-grown cells showed no oxygen consumption with DNT or MNC.

Metabolism of other nitrotoluenes. DNT-grown cells transformed 2,3-dinitrotoluene, 2,6-dinitrotoluene, 3,4-dinitrotoluene, and TNT more slowly than DNT (Table 2); no transformation was seen with any mononitrotoluene. Auxanography (20) demonstrated growth at the expense of MNC and 4-nitrocatechol as well as DNT. None of the other nitrotoluenes tested could serve as growth substrates. No attempt was made to identify the metabolites produced from the di- and trinitrotoluenes.

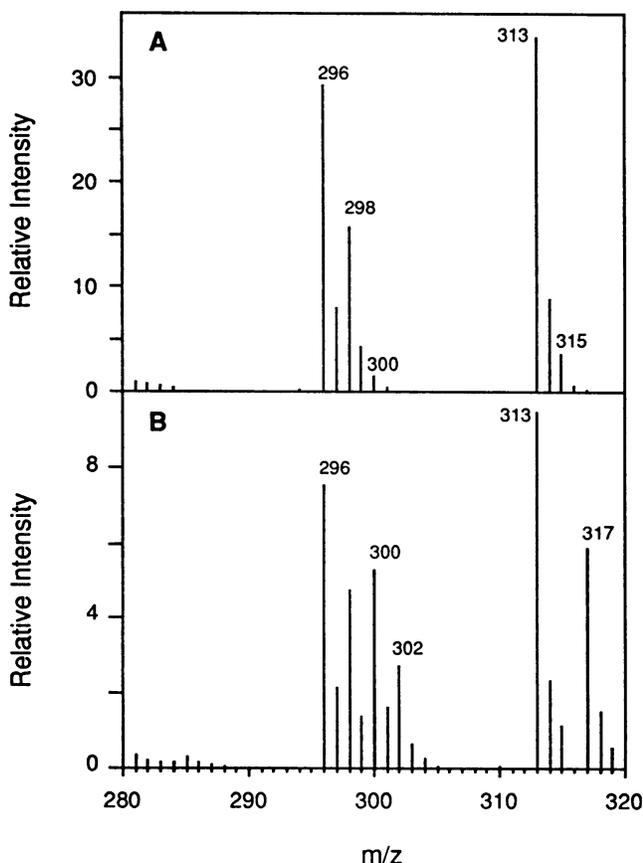


FIG. 4. $^{18}\text{O}_2$ incorporation. Mass spectra of trimethylsilyl derivative of MNC produced from DNT in the presence of air (A) or an atmosphere in which 33.7% of the oxygen was $^{18}\text{O}_2$ (B).

DISCUSSION

Oxidative removal of the nitro group from nitrophenols (25, 31) and nitrocresols (12) has been described in a number of systems. Rigorous studies with *o*- (31) and *p*-nitrophenol (25) have shown that attack by a monooxygenase results in

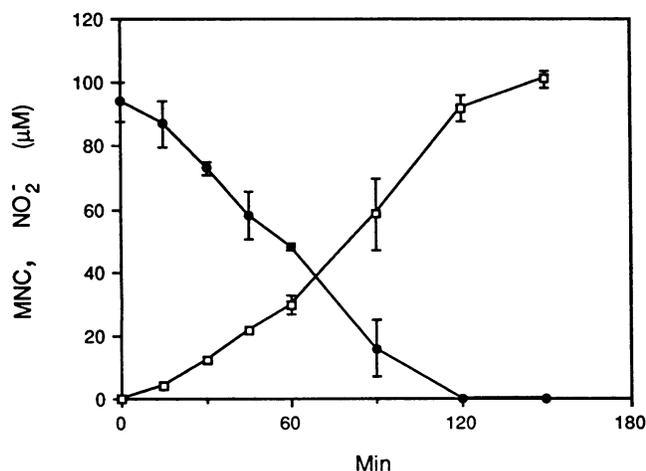


FIG. 5. Utilization of MNC (●) and release of nitrite (□) by DNT-grown cells. Points plotted are means of triplicate cultures; bars represent 1 standard deviation.

TABLE 1. Oxygen consumption by DNT-grown cells

Assay substrate ^a	Rate ($\mu\text{mol}/\text{min}$ per mg of protein)
DNT	0.026
2-Nitrotoluene	0.009
3-Nitrotoluene	<0.001
4-Nitrotoluene	<0.001
MNC	0.038
Catechol	<0.001
3-Nitrocatechol	<0.001
4-Nitrocatechol	0.014
3-Methylcatechol	<0.001
4-Methylcatechol	<0.001
3-Methyl-4-nitrophenol	0.012
1,2,4-Benzenetriol	0.018

^a Substrates were provided at 5×10^{-5} M; 1,2,4-benzenetriol was provided at 2.5×10^{-5} M.

the replacement of the nitro group by a hydroxyl group. Recently, it has been suggested that a dioxygenase attack might be involved in the displacement of a nitro group from 2,6-dinitrophenol (9). We have clearly shown here that the 4-nitro group of DNT is displaced as a result of a 4,5-dioxygenation of the parent molecule (Fig. 6). To our knowledge this is the first demonstration of removal of an aromatic nitro group by the action of a dioxygenase and the first demonstration of a dioxygenase attack on a nonpolar nitroaromatic compound. The initial dihydroxy intermediate would be expected to spontaneously eliminate nitrite and rearrange to form MNC. Although there was no evidence of accumulation of the proposed dihydroxy cyclohexadiene, the participation of such an intermediate is postulated on the basis of the $^{18}\text{O}_2$ incorporation experiment described in Fig. 4 and by analogy with other systems involving dioxygenase enzymes (11). The reaction would be analogous to that proposed for the removal of the sulfonyl group from naphthalenesulfonic acids (3). Several other functional groups are known to be eliminated from the aromatic ring either directly or indirectly by the action of dioxygenases. Removal of carboxyl (21) or acetyl (3) groups require the participation of a dehydrogenase for conversion of the dihydroxy intermediate to the corresponding catechol, whereas elimination of sulfonyl (3) and hydroxyl (3, 26) groups appears to be

TABLE 2. Transformation of nitrotoluenes by DNT-grown cells

Test substrate ^a	% Remaining		Polar metabolites ^c	
	Control ^b	Experimental		
		4 h	20 h	
2-Nitrotoluene	61	89	68	No
3-Nitrotoluene	100	100	100	No
4-Nitrotoluene	66	101	65	No
2,3-Dinitrotoluene	78	85	57	Yes
DNT	94	77	0	Yes
2,6-Dinitrotoluene	84	87	71	Yes
3,4-Dinitrotoluene	84	97	78	Yes
TNT	81	38	20	Yes
MNC	99	0	0	No
4-Nitrocatechol	99	12	0	No

^a All substrates were provided at 2.2×10^{-4} M.

^b Cell-free controls.

^c Polar metabolites were detected by HPLC.

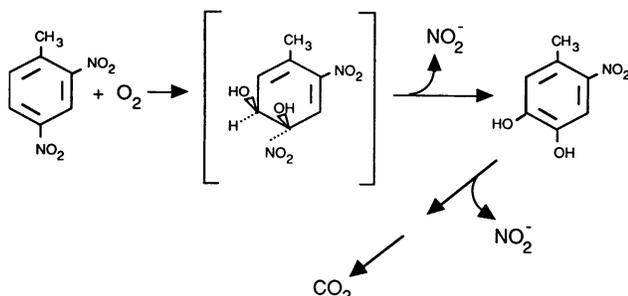


FIG. 6. Initial steps in DNT degradation pathway.

spontaneous. The mechanism of elimination of the amino group is unclear (2).

Rapid oxidation of MNC and elimination of nitrite by induced cells indicates that MNC is on the metabolic pathway for DNT degradation and not the product of a side reaction. Transient accumulation during induction for growth on DNT and the ability to serve as a growth substrate further confirm its central role in the pathway. The reactions leading to assimilation of MNC and release of the nitro group are currently under investigation.

Pseudomonas sp. strain DNT came from a mixed culture isolated from Waconda Bay, Tenn., which had previously received dilute nitroaromatic wastes from a TNT manufacturing facility (27). Preliminary experiments with other nitrotoluene isomers indicate that enzymes induced by growth on DNT can catalyze the transformation of 2,3-dinitrotoluene, 3,4-dinitrotoluene, and TNT. Additional investigation will be necessary to identify the products of these transformations. The ability of *Pseudomonas* sp. strain DNT to use DNT as a carbon source suggests that it may be useful in a treatment system for removal of nitrotoluenes from contaminated soils or from industrial waste streams. The metabolic pathway involving oxidative removal of the nitro groups avoids the accumulation of toxic amino derivatives, and mineralization of the DNT indicates that no organic residues remain. Development of a treatment system will require additional studies on the survival of the organism and on the kinetics and regulation of the catabolic reactions.

ACKNOWLEDGMENT

We thank M. Henley for assistance with MS.

REFERENCES

- Astle, M. J., and S. P. Stephenson. 1943. Polarographic investigation of hydrogen bonding. III. Nitrodihydroxybenzenes. *J. Am. Chem. Soc.* **65**:2399-2402.
- Bachofer, R., F. Lingens, and W. Schäfer. 1975. Conversion of aniline into pyrocatechol by a *Nocardia* sp.: incorporation of oxygen-18. *FEBS Lett.* **50**:288-290.
- Brilon, C., W. Beckmann, and H. Knackmuss. 1981. Catabolism of naphthalenesulfonic acids by *Pseudomonas* sp. A3 and *Pseudomonas* sp. C22. *Appl. Environ. Microbiol.* **42**:44-55.
- Budzikiewicz, H., C. Djerassi, and D. Williams. 1967. Mass spectrometry of organic compounds, p. 516. Holden-Day, Inc., San Francisco.
- Cain, R. B. 1958. The microbial metabolism of nitroaromatic compounds. *J. Gen. Microbiol.* **19**:1-14.
- Cain, R. B. 1966. Utilization of anthranilic and nitrobenzoic acids by *Nocardia opaca* and a flavobacterium. *J. Gen. Microbiol.* **42**:219-235.
- Cousin, H. 1898. 2^o Nitrohomopyrocatechine. *Ann. Chim. Phys.* **13**:537.
- Dey, S., P. Kanekar, and S. H. Godbole. 1986. Aerobic microbial degradation of *m*-dinitrobenzene. *Indian J. Environ. Health* **29**:118-128.
- Ecker, S., H.-J. Knackmuss, and C. Bruhn. 1989. Catabolism of 2,6-dinitrophenol by *Alcaligenes eutrophus* JMP222, abstr. Q-198, p. 168. Abstr. 89th Annu. Meet. Am. Soc. Microbiol. 1989. American Society for Microbiology, Washington, D.C.
- Fernando, T., J. A. Bumpus, and S. D. Aust. 1990. Biodegradation of TNT (2,4,6-trinitrotoluene) by *Phanerochaete chrysosporium*. *Appl. Environ. Microbiol.* **56**:1666-1671.
- Gibson, D. T., and V. Subramanian. 1984. Microbial degradation of aromatic hydrocarbons, p. 181-252. In D. T. Gibson (ed.), *Microbial degradation of organic compounds*. Marcel Dekker, Inc., New York.
- Gundersen, K., and H. L. Jensen. 1956. A soil bacterium decomposing organic nitro-compounds. *Acta Agric. Scand.* **6**:100-114.
- Hallas, L. E., and M. Alexander. 1983. Microbial transformation of nitroaromatic compounds in sewage effluent. *Appl. Environ. Microbiol.* **45**:1234-1241.
- Keith, L. H., and W. A. Telliard. 1979. Priority pollutants. I. A perspective view. *Environ. Sci. Technol.* **13**:416-423.
- Kulpa, C., and M. Wilson. 1991. Degradation of trinitrotoluene by a mixed microbial culture isolated from soil, abstr. A-159, p. 303. Abstr. 91st Annu. Meet. Am. Soc. Microbiol. 1991. American Society for Microbiology, Washington, D.C.
- Liu, D., K. Thomson, and A. C. Anderson. 1984. Identification of nitroso compounds from biotransformation of 2,4-dinitrotoluene. *Appl. Environ. Microbiol.* **47**:1295-1298.
- McCormick, N. G., J. H. Cornell, and A. M. Kaplan. 1978. Identification of biotransformation products from 2,4-dinitrotoluene. *Appl. Environ. Microbiol.* **35**:945-948.
- McCormick, N. G., F. F. Feeherry, and H. S. Levinson. 1976. Microbial transformation of 2,4,6-trinitrotoluene and other nitroaromatic compounds. *Appl. Environ. Microbiol.* **31**:949-958.
- Neumeier, W., R. Haas, and E. V. Löw. 1989. Microbieller Abbau von Nitroaromaten aus einer ehemaligen Sprengstoffproduktion. *Forum Staedte-Hyg.* **37**:32-37.
- Parke, D., and L. N. Ornston. 1984. Nutritional diversity of *Rhizobiaceae* revealed by auxanography. *J. Gen. Microbiol.* **130**:1743-1750.
- Reiner, A. M. 1972. Metabolism of aromatic compounds in bacteria. Purification and properties of the catechol-forming enzyme, 3,5-cyclohexadiene-1,2-diol-1-carboxylic acid (NAD⁺) oxidoreductase (decarboxylating). *J. Biol. Chem.* **247**:4960-4965.
- Smbert, R. M., and N. R. Krieg. 1981. General characterization, p. 409-443. In P. Gerhardt, R. G. E. Murray, R. N. Costilow, E. W. Nester, W. A. Wood, N. R. Krieg, and G. B. Phillips (ed.), *Manual of methods for general bacteriology*. American Society for Microbiology, Washington, D.C.
- Smith, P. K., R. I. Krohn, G. T. Hermanson, A. K. Mallia, F. H. Gartner, M. D. Provenzano, E. K. Fujimoto, N. M. Goeke, B. J. Olson, and D. C. Klenk. 1985. Measurement of protein using bicinchoninic acid. *Anal. Biochem.* **150**:76-85.
- Spain, J. C., and S. F. Nishino. 1987. Degradation of 1,4-dichlorobenzene by a *Pseudomonas* sp. *Appl. Environ. Microbiol.* **53**:1010-1019.
- Spain, J. C., O. Wyss, and D. T. Gibson. 1979. Enzymatic oxidation of *p*-nitrophenol. *Biochem. Biophys. Res. Commun.* **88**:634-641.
- Spain, J. C., G. J. Zylstra, C. K. Blake, and D. T. Gibson. 1989. Monohydroxylation of phenol and 2,5-dichlorophenol by toluene dioxygenase in *Pseudomonas putida* F1. *Appl. Environ. Microbiol.* **55**:2648-2652.
- Spanggord, R. J., B. Gibson, R. Keck, and D. Thomas. 1982. Effluent analysis of wastewaters generated in the manufacture of 2,4,6-trinitrotoluene. I. Characterization study. *Environ. Sci. Technol.* **16**:229-234.
- Spanggord, R. J., T. Mill, T. Chou, W. Mabey, J. Smith, and S. Lee. 1980. Environmental fate studies on certain munition

- wastewater constituents. Laboratory studies. Report no. ADA099256. SRI International, Menlo Park, Calif.
29. **Tewfik, M. S., and W. C. Evans.** 1966. The metabolism of 3,5-dinitro-*o*-cresol (DNOC) by soil microorganisms. *Biochem. J.* **99**:31–32.
 30. **Unkefer, P. J., M. A. Alvarez, J. L. Hanners, C. J. Unkefer, M. Stenger, and E. A. Margiotta.** 1990. Bioremediation of explosives, p. 307–326. JANNAF Safety and Environmental Protection Subcommittee Workshop: alternatives to open burning/open detonation of propellants and explosives. Chemical Propulsion Information Agency, Laurel, Md.
 31. **Zeyer, J., and P. C. Kearney.** 1984. Degradation of *o*-nitrophenol and *m*-nitrophenol by a *Pseudomonas putida*. *J. Agric. Food Chem.* **32**:238–242.