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Reduction and Acetylation of 2,4-Dinitrotoluene by a *Pseudomonas aeruginosa* Strain

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Aerobic and anoxic biotransformation of 2,4-dinitrotoluene (DNT) was examined by using a *Pseudomonas aeruginosa* strain isolated from a plant treating propellant manufacturing wastewater. DNT biotransformation in the presence and absence of oxygen was mostly reductive and was representative of the type of cometabolic transformations that occur when a high concentration of an easily degradable carbon source is present. *P. aeruginosa* reduced both nitro groups on DNT, with the formation of mainly 4-amino-2-nitrotoluene and 2-amino-4-nitrotoluene and small quantities of 2,4-diaminotoluene. Acetylation of the arylamines was a significant reaction. 4-Acetamide-2-nitrotoluene and the novel compounds 2-acetamide-4-nitrotoluene, 4-acetamide-2-aminotoluene, and 2,4-diacetamidetoluene were identified as DNT metabolites. The biotransformation of 2,4-diaminotoluene to 4-acetamide-2-aminotoluene was 24 times faster than abiotic transformation. 2-Nitrotoluene and 4-nitrotoluene were also reduced to their corresponding toluidines and then acetylated. However, the yield of 4-acetamidetoluene was much higher than that of 2-acetamidetoluene, demonstrating that acetylation at the position *para* to the methyl group was favored.

The presence of nitrotoluenes in the environment is primarily the result of their use in the manufacture of pesticides and dyes and as explosives and propellants (12). There are two general mechanisms for the biological transformation of nitroaromatic compounds, namely, oxidation and reduction (28). In oxidative reactions, molecular oxygen is a required reactant, and oxygenase (32) or peroxidase (34, 36) enzymes mediate the cleavage of the aromatic ring, resulting in mineralization of the compounds. A more common reaction affecting nitroaromatic compounds is their reduction to arylamines (15), which can occur under both aerobic and anaerobic conditions. Further transformation of the arylamines is also possible through mechanisms such as hydrolytic deamination (4), acetylation (9, 23, 29), reductive deamination (23, 35), and cyclization (11, 23).

During aerobic bioremediation and treatment of nitroaromatic-contaminated wastewaters and soils, competition between oxidative and reductive transformations is of paramount importance in determining the success of the treatment. While in some cases reduction of nitroaromatic compounds to the corresponding arylamines is a required initial step before oxidative biotransformation and mineralization take place (5, 10, 36), reduction can also result in the formation of more refractory by-products, diminishing the extent of mineralization. For example, during the degradation of 2,4-dinitrotoluene (DNT) by *Phanerochaete chrysosporium*, a portion of the original DNT is transformed to 4-amino-2-nitrotoluene (4Am2NT) and 2,4-diaminotoluene (DAT), which are not intermediates in the mineralization pathway (36). This may partially explain the low mineralization yields observed.

Competition between reductive and oxidative transformations is of particular importance in the aerobic treatment of DNT-contaminated wastewaters from propellant manufacturing, where high concentrations of an easily degradable organic source such as ethanol (17, 24, 31) appears to favor the cometabolic reduction of DNT. Freedman et al. (7) illustrated this effect in cultures enriched from the biological treatment system at the Radford Army Ammunition Plant (RAAP). When cultures were grown on DNT as the only source of organic carbon, 100% of the DNT was oxidatively transformed, but when the same culture was incubated with ethanol as the main organic carbon source, only 59% of the DNT was directly oxidized, and reduced products such as 2-amino-4-nitrotoluene (2Am4NT) and 4Am2NT accumulated transiently (7). In addition, 2Am4NT and 4Am2NT are frequently detected during routine analysis of the wastewater effluent from RAAP (20). Because the treatment plant at RAAP receives high concentrations of easily degradable organic substrates (in addition to DNT), it facilitates the growth of microorganisms with a variety of metabolic capabilities, and therefore, the fate of DNT and the efficiency of treatment depend on (i) how efficiently DNT-oxidizing microorganisms compete with other organisms that reductively transform DNT and (ii) the ability of organisms in the culture to transform and mineralize the reduced products of DNT.

Our primary objective was to investigate the fate of the portion of DNT that is reductively transformed during aerobic treatment at RAAP. We have reported previously (7) on the development of aerobic cultures enriched from the RAAP treatment plant that are able to oxidize 2Am4NT, 4Am2NT, and DAT, which are the usual products of the reductive transformation of DNT (19, 36). More recently, we reported the identification of mononitrotoluenes, acetylated aminotoluenes, and 6-nitroindazole as products from the reductive transformation of DNT in an anoxic culture enriched from RAAP (23). In this paper, we report on the aerobic reduction and acetylation of DNT and several DNT metabolites by a strain of *Pseudomonas aeruginosa* isolated from the anoxic enrichment culture. This activity is important in understanding the overall fate of DNT during aerobic treatment of munition wastes at RAAP.

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MATERIALS AND METHODS

Chemicals. All of the commercially available aromatic compounds purchased were of the highest purity possible. Solvents such as methanol, ethanol, and

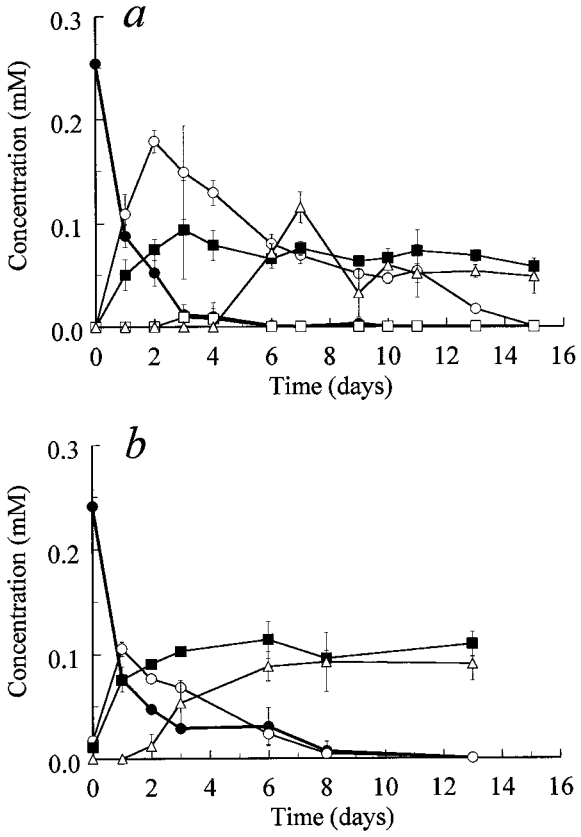

FIG. 1. Biotransformation of DNT by *P. aeruginosa* under aerobic (a) and anoxic (b) conditions. Error bars indicate 1 standard deviation of duplicate samples. Symbols: ●, DNT; ○, 4Am2NT; ■, 2Am4NT; △, 4Acm2NT; □, DAT.

methylene chloride were all high-performance liquid chromatography (HPLC) grade. 2-Acetamide-4-nitrotoluene (2Acm4NT), 4-acetamide-2-nitrotoluene (4Acm2NT), 2-acetamidetoluene, 4-acetamidetoluene, and 2,4-diacetamidetoluene were synthetically produced by reacting the corresponding aminotoluenes with acetic anhydride by the procedure of McCormick et al. (18) for the synthesis of acetamidenitrotoluenes. Purification of the products was carried out by recrystallization with mixtures of ethanol and water (30).

Uniformly labeled [^{14}C]DNT with a specific activity of 7.0 mCi/mmol and a purity greater than 97.8% was purchased from NEN Research Products (Boston, Mass.). A stock solution containing 4.4×10^6 dpm/ml (52.6 mg of DNT per liter) was prepared by dissolving the tracer in 500 ml of water. The purity of the radiotracer was evaluated by injection into the HPLC and collection of the eluent at 0.5-min intervals. The radioactivity corresponding to the DNT peak accounted for 99.2% of the total tracer recovered. Total recovery (amount collected relative to amount injected) was 98.0%.

Culture conditions and media. An initial anoxic (nitrate-reducing) enrichment culture was developed with inoculum from the rotating biological contactors at RAAP as described previously (23). DNT is the main nitroaromatic compound in their waste stream (1, 13), so the inoculum was well acclimated. The purpose of the enrichment was twofold: first, to evaluate the biotransformation and possible mineralization of DNT under anoxic conditions (23), and, second, to select for microorganisms capable of cometabolic DNT reduction but with limited ability to oxidatively transform DNT, since oxidation requires molecular oxygen (33).

Microorganisms were grown in a mineral medium that contained 70 mM K_2HPO_4, 10 mM NaH_2PO_4, 24 mM $NaHCO_3$, 0.41 mM $MgSO_4$, 68 μM $CaCl_2$, 50 μM $FeCl_2$, 12.6 μM $CoCl_2$, 10 μM Na_2MoO_4, 10 μM Na_2SeO_3, 10.1 μM $MnCl_2$, 6.3 μM $NiCl_2$, 1.5 μM $ZnCl_2$, and 0.8 μM H_3BO_4.

P. aeruginosa was grown aerobically at 35°C in mineral medium amended with NH_4Cl (117 mM) and ethanol (34 mM). Ammonia was supplied in excess to allow several additions of ethanol in a single experiment. Atmospheric oxygen served as the electron acceptor. At the point when DNT was added, the optical density (600 nm) of the culture was 0.56 (samples diluted fourfold), corresponding to a total dry weight of 2.3 g/liter. Anoxically, *P. aeruginosa* was grown at 35°C in mineral medium amended with ethanol (34 mM) as the electron donor and carbon source and nitrate (71 mM) as the electron acceptor and nitrogen source. The headspace of the bottles was flushed with helium or nitrogen gas before the bottles were sealed. The absence of oxygen was confirmed by gas chromatographic (GC) analysis of the headspace (described below). Ethanol and nitrate (as HNO_3) were added each time they were depleted. The pH was maintained between 6.5 and 7.8. The cultures were incubated quiescently, in closed glass bottles, in an anaerobic glove box. Prior to the addition of DNT, bacterial dry weight was 1.75 g/liter, with an optical density of 0.25 (fourfold dilution).

Isolation and identification of *P. aeruginosa*. Samples from the anoxic enrichment cultures were grown in solid plates (mineral medium plus 17.8 g of agar per liter). Nitrate was added as the nitrogen source and electron acceptor during anoxic incubations. DNT (0.274 mM) and ethanol (34 mM) were added aseptically (from a stock solution containing 25 g of DNT per liter of ethanol) immediately after autoclaving. The plates were incubated at 35°C, either aerobically or anoxically (in an anaerobic glove box with an atmosphere of 99% N_2 and 1% H_2).

After purification of the fastest-growing microorganisms from the aerobic and anoxic plates, they were identified by GC-FAME and Biolog analyses (Microbe Inotech Laboratories, Inc., St. Louis, Mo.) as *P. aeruginosa*. Microscopic observations indicated agreement between the aerobic and anoxic plate analyses: a rod-shaped, motile, gram-negative bacterium that grew as single cells was found.

Analytical methods. The quantitative analysis of nitroaromatics and aminoaromatics was performed by GC on a DB-1 column with a flame ionization detector (22). Sample preparation for GC analysis included mixing equal volumes of culture and methanol prior to filtration to minimize solute losses (14), followed by extraction with methylene chloride (22, 23).

HPLC gradient separations of nitroaromatics and aminoaromatics were made on a reversed-phase column (Hi-Pore RP-318; Bio-Rad Laboratories, Richmond, Calif.) with an aqueous buffer-methanol eluent and detection by UV at 254 nm (22).

Identification of methylene chloride-extractable DNT metabolites was performed with the following two GC-mass spectrometry (GC-MS) systems: (i) a Hewlett-Packard 5971 Series mass selective detector connected to a Hewlett-Packard 5890 Series II GC, equipped with an HP-1 capillary column (12 m by 0.2 mm by 0.33 μm); and (ii) a VG 70-VSE connected to a Hewlett-Packard 5890 GC, equipped with a DB-5 capillary column (J&W Scientific).

The concentration of ethanol in cultures was quantified by transferring a 5-ml sample to a 12-ml serum vial, sealing with a gray butyl rubber septum, allowing the ethanol to equilibrate between the aqueous and gaseous phases, and then analyzing a headspace sample by GC (22). Nitrate was determined by ion-exchange chromatography with an IC-Pak A column (Waters, Milford, Mass.) at 25°C, with detection by UV at 214 nm (22). The presence of oxygen in the headspace of cultures incubated in sealed glass bottles was monitored by GC, with an 80/100 mesh Molecular Sieve 5A column (3.2 mm by 2.4 m, stainless steel) on a Series 580 GC (Gow-Mac Instruments Co., Bridgewater, N.J.) equipped with a thermal conductivity detector. Bacterial density was determined gravimetrically by filtering samples (0.2-μm-pore-size filter; Millipore) and drying them overnight at 103°C and by determining the optical density at 600 nm. Samples were diluted fourfold with distilled water before measurement of optical density. Analysis of radiolabeled samples was made with Tri-Carb liquid scintillation analyzer (Packard Instruments Co., Downers Grove, Ill.). Ecoscint scintillation cocktail (National Diagnostic, Manville, N.J.) was used.

RESULTS

Biotransformation of DNT by *P. aeruginosa*. The ability of *P. aeruginosa* to biotransform DNT was tested under aerobic and anoxic (nitrate-reducing) conditions. Figure 1a presents a typical aerobic result. After 4 days of incubation, 96% of the initial DNT was transformed, with 4Am2NT, 2Am4NT, and DAT accounting for 53, 32, and 3% of the DNT by-products, respectively. Very little subsequent transformation of 2Am4NT occurred. In contrast, 4Am2NT and DAT were completely transformed, coincident with the accumulation of 4Acm2NT and traces of acetamide-aminotoluene. This novel acetylated product of DAT was identified by GC-MS (Fig. 2a; the mass spectrum was similar to that of DAT, with the exception of the M^+ at 164). No accumulation of 2Acm4NT or 2,4-diacetamidetoluene was observed. After 15 days of incubation, only 43% of the original DNT was accountable, mainly as 4Acm2NT and 2Am4NT.

The anoxic fate of DNT was similar to that under aerobic conditions. As shown in Fig. 1b, there was an initial reduction of DNT to 2Am4NT, which then persisted, and 4Am2NT, which was acetylated to 4Acm2NT. Accumulation of DAT or acetamide-aminotoluene was not observed. After 13 days of incubation, all of the DNT was biotransformed, with 2Am4NT

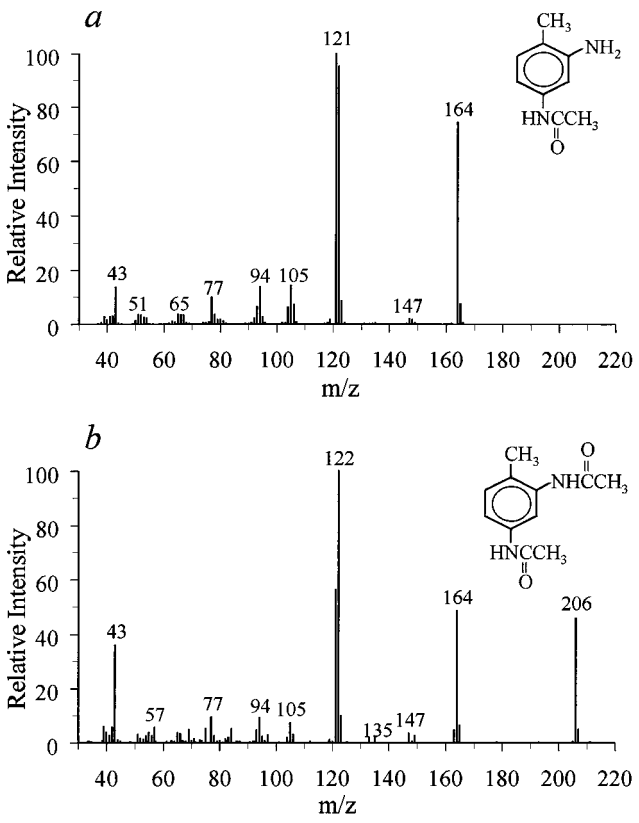

FIG. 2. Mass spectra of the metabolites identified as 4-acetamide-2-aminotoluene (a) and 2,4-diacetamidetoluene (b).

and 4Acm2NT accounting for 44 and 36% of the original DNT, respectively, and 20% not accounted for.

Since the accumulation of products in the aerobic and anoxic cultures did not account for 100% of the initial DNT added, uniformly labeled [^{14}C]DNT was added (20,800 dpm/ml) to determine the fate of the unaccounted DNT. Table 1 summarizes the results after 32 days of aerobic incubation with the ^{14}C tracer. Losses by volatilization accounted for only 3.7% of the original tracer. The possibility of $^{14}CO_2$ formation was evaluated with filtered samples by lowering the pH, stripping with nitrogen gas, and then comparing the radioactivity remaining with the ^{14}C in the initial samples. The decrease in ^{14}C following acidification and stripping was statistically insignificant, indicating that minimum mineralization of DNT occurred. Losses during filtration of samples accounted for 7.5% of the total tracer. After rinsing the filters three times with a phosphate buffer, only 20% of this fraction still remained with the filter.

The soluble fraction of the DNT by-products (soluble materials recovered from the filters by washing were excluded to avoid dilution of the samples) was characterized by HPLC and scintillation counting of the column effluent, collected at 0.5-min intervals. The ^{14}C recovery (disintegrations per minute in all fractions relative to disintegrations per minute of the filtered sample) was always greater than 90%. The eluent fractions were separated into the following five subgroups (Table 1), depending on the relative hydrophobicity and their retention times. (i) The first subgroup included unretained hydrophilic metabolites, which were poorly extracted in methylene chloride and could not be identified by GC-MS. (ii) The less hydrophilic subgroup included compounds with a retention time between 5 and 10 min and an efficiency of extraction in methylene chloride of 45%; DAT corresponded to this group, but GC measurements indicated no significant accumulation of this compound. (iii) The third subgroup included 2Am4NT. GC measurements indicated a concentration of 2Am4NT of 27% ± 2% of the original DNT, which is in good agreement with the ^{14}C results. (iv) The fourth subgroup included 4Acm2NT and DNT, which coeluted in the HPLC with a retention time of 13 min. GC measurements indicated that most of the DNT was already transformed and that the accumulation of 4Acm2NT corresponded to about 33% of the original DNT. Therefore, it appears as though an unidentified metabolite coeluted with 4Acm2NT and DNT. (v) The last subgroup included very hydrophobic metabolites with retention times greater than 15 min. This retention time corresponded to that of azoxynitrotoluenes. However, their presence in the cultures could not be confirmed by GC-MS.

Biotransformation of reduced DNT metabolites. DAT, an important metabolite from the reductive transformation of DNT (2, 23), was found only in small concentrations in the experiments with *P. aeruginosa*. Nevertheless, traces of a novel acetylated product of DAT were identified from the aerobic cultures. To confirm that transformation of DAT was biologically mediated, *P. aeruginosa* was incubated with DAT as the only aromatic substrate and ethanol as the main electron donor. Figure 3 shows that DAT was acetylated rapidly. No other metabolites were detected during the transformation.

A sterile control with DAT dissolved in mineral medium (Fig. 3) confirmed that *P. aeruginosa* mediated the acetylation of DAT. In this experiment, DAT was very slowly transformed (2.7% disappearance in 5 days). After 5 days, the headspace of the control bottle was replaced with pure oxygen. This resulted in an increased transformation rate, and after 5 more days, the bottle had a noticeable change in color, from clear to dark brown, and a dark precipitate started to be visible. In the absence of oxygen (sealed bottles flushed with nitrogen gas), DAT was not abiotically transformed and the bottle contents remained colorless.

It was not possible to directly determine which acetamide-aminotoluene isomer was produced during the acetylation of DAT by *P. aeruginosa*. We were able to chemically synthesize both isomers but were not able to purify them. Indirect evidence such as the formation of 4Acm2NT but not 2Acm4NT from DNT suggested that acetylation preferentially occurs at the *para* position. To evaluate this hypothesis, experiments with 2-nitrotoluene and 4-nitrotoluene were conducted. Figure 4 shows that both compounds were rapidly reduced to the corresponding toluidines but only 4-acetamidetoluene was produced in significant amounts. 2-Acetamidetoluene was also

TABLE 1. Mass balance on metabolites from aerobic biotransformation of DNT

Metabolite fraction (group and subgroup)	% ^{14}C (mean ± SD)[a]	
	Group	Subgroup
Lost by volatilization	3.7 ± 0.4	
Lost by filtration	7.5 ± 2.5	
Soluble	88.7 ± 2.4	
(i) Unretained		7.6 ± 0.2
(ii) Less hydrophilic		8.7 ± 1.1
(iii) 2Am4NT		29.9 ± 2.2
(iv) 4Acm2NT		42.0 ± 2.6
(v) Very hydrophobic		3.2 ± 1.4

[a] Mean ± standard deviation of duplicate samples.

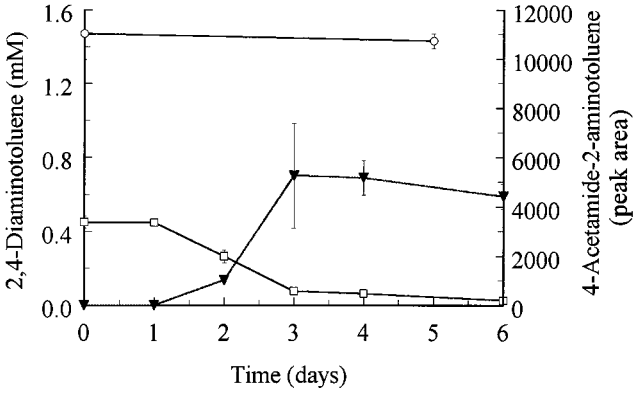

FIG. 3. Biotransformation of DAT by *P. aeruginosa*. Error bars indicate 1 standard deviation of duplicate samples. Symbols: ○, DAT, sterile medium bottle; □, DAT, live culture; ▼, 4-acetamide-2-aminotoluene, live culture.

formed but at a much lower rate. On this basis, there is a reasonably high degree of certainty that the acetylated product of DAT was 4-acetamide-2-aminotoluene.

Additional experiments were set up with 4Am2NT or 2Am4NT as the only aromatic substrate (Fig. 5). The rapid acetylation of 4Am2NT was confirmed. In addition, a small yield of DAT was observed, indicating that reduction of the nitro group occurred; however, reduction was not as efficient as acetylation. 4Acm2NT was further reduced to 4-acetamide-2-aminotoluene, which persisted in the culture. 2,4-Diacetamidetoluene, the product of complete reduction and acetylation, was also detected in trace amounts (Fig. 2b). 2Am4NT was transformed very slowly. During its degradation, only traces of 2Acm4NT, DAT, and 4-acetamide-2-aminotoluene were detected.

Table 2 summarizes the results obtained with transformation of various substrates by *P. aeruginosa* along with metabolites identified by GC-MS. DNT, DAT, and 4Am2NT were transformed an order of magnitude faster than were 2Am4NT and the mononitrotoluene isomers.

DISCUSSION

The biological treatment process at RAAP contains organisms capable of oxidizing and mineralizing DNT (7). Oxidation results in the stoichiometric release of nitrate (7), possibly following the pathway previously described for a strain of *Pseudomonas* sp. (33). However, the presence of high concentrations of ethanol in the wastewater alters the extent of oxidation in two ways, namely, (i) it allows the growth of other organisms that utilize ethanol as the main carbon source and reductively transform DNT by cometabolism and (ii) organisms able to oxidize DNT may choose alternative pathways, since DNT is not the only source of carbon and energy available. The overall result is that a significant fraction of the DNT is cometabolically reduced rather than directly oxidized (7).

The strain of *P. aeruginosa* reported here grows very rapidly in the presence of high concentrations of ethanol and is very effective at reducing DNT. Its presence in the RAAP treatment plant explains earlier results by Freedman et al. in which aerobic enrichments from RAAP directly oxidized DNT in the absence of additional carbon sources but transformed DNT by a combination of oxidative (59%) and reductive (41%) pathways when ethanol was supplied as the main carbon source (7). The initial products of DNT transformation by *P. aeruginosa* were 4Am2NT and 2Am4NT (Fig. 6). The microflora at RAAP contains organisms able to utilize these compounds as sole sources of carbon and energy (7), and presumably, a fraction of them is degraded in that way. *P. aeruginosa* also transforms these compounds in a variety of ways. 4Am2NT is rapidly acetylated to 4Acm2NT, while 2Am4NT is very slowly transformed to DAT and 2Acm4NT. Formation of DAT from 4Am2NT by *P. aeruginosa* also occurs (Fig. 6) but with a very low yield (Fig. 5).

Complete reduction of polynitrated toluenes to the corresponding aminotoluenes usually requires a low redox potential (26), and therefore, high DAT yields are not typically expected under aerobic conditions. Accordingly, only small amounts of DAT were detected in the aerobic *P. aeruginosa* cultures. Additional reasons why there was no significant accumulation of DAT include the following: first, *P. aeruginosa* rapidly acetylated DAT, resulting in the formation of 4-acetamide-2-aminotoluene (Fig. 3); second, transformation of 2Am4NT by *P. aeruginosa* was very poor; and third, acetylation of the amine group in 4Am2NT occurred much faster than reduction of the nitro group. 4-Acetamide-2-aminotoluene was also transformed, as evidenced by the identification of 2,4-diacetamidetoluene in the cultures that received 4Am2NT (Table 2). Detection and identification of 2,4-diacetamidetoluene were difficult because it eluted very late on the GC column used (retention time, greater than 30 min), and as a result of band broadening in the column, it required molar concentrations much higher than the initial DNT concentrations used in the experiments before it could be detected (22). We were able to

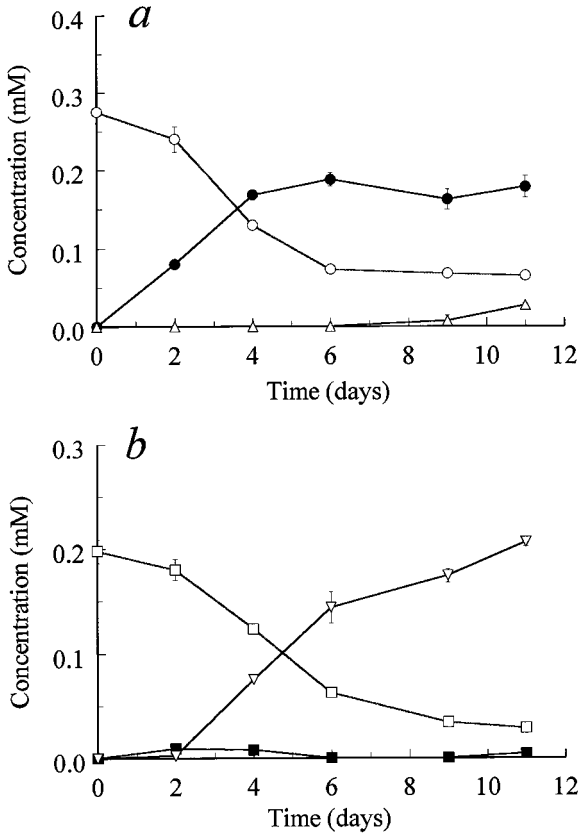

FIG. 4. Biotransformation of 2-nitrotoluene (a) and 4-nitrotoluene (b) by *P. aeruginosa*. Error bars indicate 1 standard deviation of duplicate samples. Symbols: ○, 2-nitrotoluene; ●, 2-aminotoluene; △, 2-acetamidetoluene; □, 4-nitrotoluene; ■, 4-aminotoluene; ▽, 4-acetamidetoluene.

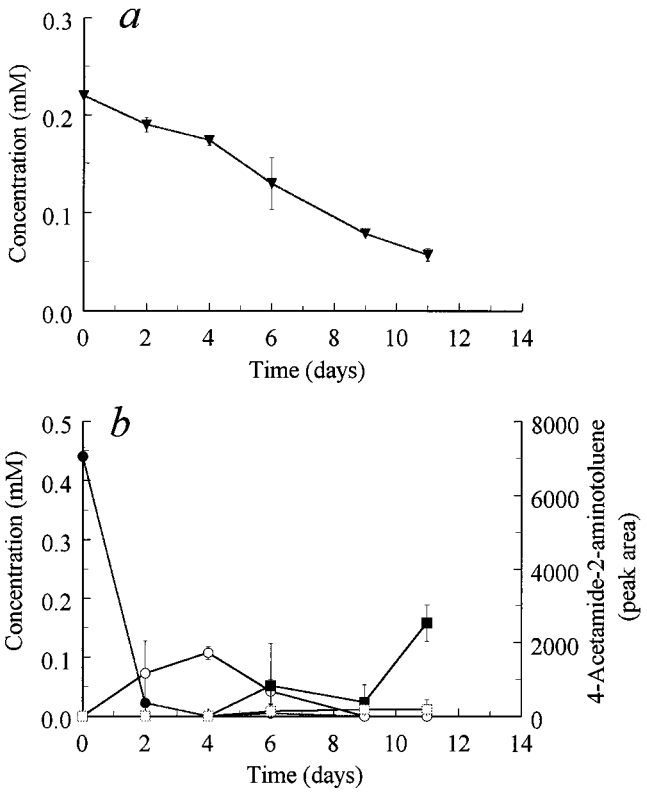

FIG. 5. Biotransformation of 2Am4NT (a) and 4Am2NT (b) by *P. aeruginosa*. Error bars indicate 1 standard deviation of duplicate samples. Symbols: ▼, 2Am4NT; ●, 4Am2NT; ○, 4Acm2NT; ■, 4-acetamide-2-aminotoluene; ⬚, DAT.

detect the compound only after extraction and concentration of the organic phase and the use of a shorter GC column (a 12-m HP-1 capillary column instead of a 30-m DB-1 megabore).

2-Acetamide-4-aminotoluene was never detected in the cultures, probably because acetylation is much more efficient at the *para* position, and most of the DAT formed would have been transformed to 4-acetamide-2-aminotoluene. 2-Acetamide-4-aminotoluene could also be formed from the reduction of 2Acm4NT, but since this compound was only produced in very small amounts from 2Am4NT, any further transformation would not have been measurable in our experiments.

Analysis of ^{14}C-labeled metabolites from [^{14}C]DNT (Table 1) provides some insight into the characteristics of the still-unidentified by-products. A small fraction of the ^{14}C label was retained during filtration (7.5%). Only 20% of this fraction was associated with insoluble compounds. The rest was washed out of the filters after rinsing with a phosphate buffer. This insoluble ^{14}C fraction may be the result of either the formation of insoluble compounds due to conjugation and polymerization reactions (28) or the binding of DNT metabolites (e.g., hydroxylamine and nitroso compounds) to cellular components (3). Assimilation of ^{14}C for cell synthesis was unlikely since there was no evidence of DNT mineralization or its use as a carbon source.

The majority of the ^{14}C in the soluble fraction was associated with the accumulation of 2Am4NT and 4Acm2NT (Table 1). However, about 25% of the ^{14}C tracer included unidentified soluble metabolites with diverse HPLC retention times. Production of hydrophilic materials from degradation of nitroaromatics has often been reported (6, 8, 23), but none of these metabolites has been identified thus far. The small fraction of very hydrophobic materials (3.2%) may be related to the formation of azoxynitrotoluene compounds. A preliminary evaluation of the HPLC retention times of three possible azoxynitrotoluene compounds formed from DNT indicated retention times similar to those of the very hydrophobic fraction. However, GC-MS analyses of this fraction did not confirm the HPLC results. Finally, the difference between ^{14}C and GC measurements indicated the possibility of unidentified ^{14}C metabolites coeluting with 4Acm2NT and DNT.

The coelution of DNT with 4Acm2NT and possibly other metabolites has a practical implication, since HPLC is the method of choice when analyzing the quality of treatment at RAAP (20). Current regulations have set a DNT discharge limit of 0.113 mg/liter (2). Routine measurements of effluent quality indicate that the current treatment facility is unable to consistently meet this limit (16, 17, 20). However, if 4Acm2NT is produced from the reductive transformation of DNT, it is possible that effluent DNT concentrations determined by HPLC are overestimated.

The biotransformation of DAT to 4-acetamide-2-aminotoluene, and ultimately to 2,4-diacetamidetoluene, deserves special attention, since the fate of the by-products of complete reduction of nitrotoluenes is still an open question. 2,4,6-Triaminotoluene, the product of complete reduction of 2,4,6-trinitrotoluene, is unstable in the presence of oxygen (28). It rapidly undergoes polymerization reactions. Although not as reactive as 2,4,6-triaminotoluene, DAT is also abiotically transformed. In sterile controls with DAT dissolved in mineral medium, the rate of DAT transformation was accelerated when the oxygen concentration was increased. In the absence of oxygen, DAT was not abiotically transformed. The rate of acetylation of DAT by *P. aeruginosa* was 24 times faster than the abiotic transformation, indicating that biological acetylation is a likely mechanism involved in the transformation of polyaminotoluenes. Biological acetylation could explain the transformation

TABLE 2. Products detected during the aerobic transformation of nitroaromatics and aminoaromatics by *P. aeruginosa* and maximum transformation rates

Aromatic substrate	Maximum rate (μM/day)	Specific rate[a] (μM/mg/day)	Product(s)
DNT	160	0.042	2Am4NT, 4Am2NT, DAT, 4Acm2NT, 4-acetamide-2-aminotoluene
DAT	190	0.086	4-Acetamide-2-aminotoluene
4Am2NT	210	0.054	DAT, 4Acm2NT, 4-acetamide-2-aminotoluene, 2,4-diacetamidetoluene
2Am4NT	15	0.0027	DAT, 4-acetamide-2-aminotoluene, 2Acm4NT
4-Nitrotoluene	30	0.0070	4-Aminotoluene, 4-acetamidetoluene
2-Nitrotoluene	42	0.011	2-Aminotoluene, 2-acetamidetoluene

[a] Specific rate was calculated by using the average biomass concentration during the degradation experiment.

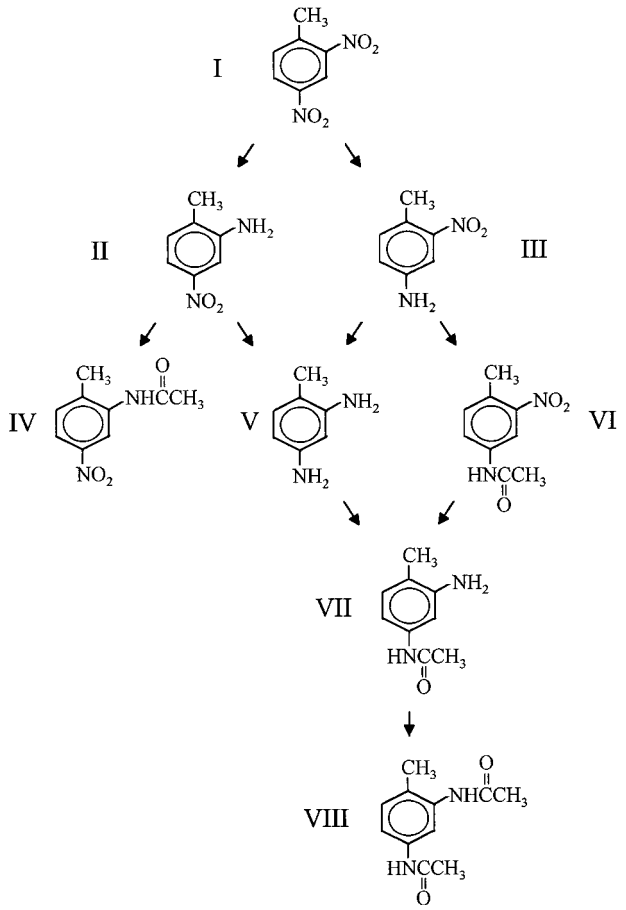

FIG. 6. Proposed pathway for the reduction and acetylation of DNT by *P. aeruginosa*. I, DNT; II, 2Am4NT; III, 4Am2NT; IV, 2Acm4NT; V, DAT; VI, 4Acm2NT; VII, 4-acetamide-2-aminotoluene; VIII, 2,4-diacetamidetoluene.

of 2,4,6-triaminotoluene to unknown products observed by Preuss et al. (25) with *Pseudomonas* organisms under anoxic conditions. Although we did not detect 4-acetamide-2-aminotoluene in the anoxic experiments (mainly because DAT did not significantly accumulate), the rates of acetylation of 4Am2NT were similar under aerobic and anoxic conditions, demonstrating that acetylation was not dependent on the presence of oxygen or the redox condition. In addition, it has now been demonstrated that reduced products of 2,4,6-trinitrotoluene can also be acetylated. Gilcrease and Murphy (9) recently reported the formation of 4-*N*-acetylamino-2-amino-6-nitrotoluene under anoxic conditions by *P. fluorescens.*

Pseudomonads are a versatile group of organisms commonly found in natural systems. While there are several examples of *Pseudomonas* spp. isolated from nitroaromatic-contaminated sites that are able to oxidize and mineralize nitroaromatic compounds (10, 27, 33), the results from this and other studies indicate that there are also *Pseudomonas* spp. which are very efficient at cometabolically reducing the nitro groups (9, 21, 29). Therefore, in a mixed substrate environment, the success of aerobic remediation depends on the ability of microorganisms to oxidize nitroaromatic compounds as well as their reduced products. The acetylation of arylamines by various *Pseudomonas* spp. (9, 21, 29) is a reaction that competes with other biological and abiotic reactions, all of which can impact the fate of the reduced products from nitrotoluenes. Whether acetylation of arylamines improves or decreases the chances for aerobic remediation of nitroaromatic-contaminated soils and wastewaters depends on the fate of the acetylated compounds, which needs further investigation.

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