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Bioconversion of 2,4-Diamino-6-Nitrotoluene to a Novel Metabolite under Anoxic and Aerobic Conditions

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Under nitrate-reducing, nongrowth conditions, a *Pseudomonas fluorescens* species reduced 2,4,6-trinitrotoluene to aminodinitrotoluenes, which were then further reduced to diaminonitrotoluenes. 2,4-Diamino-6-nitrotoluene (2,4-DANT) was further transformed to a novel metabolite, 4-*N*-acetylamino-2-amino-6-nitrotoluene (4-*N*-AcANT), while 2,6-diamino-4-nitrotoluene (2,6-DANT) was persistent. Efforts to further degrade 2,4-DANT and 2,6-DANT under aerobic, nitrogen-limited conditions were unsuccessful; 2,6-DANT remained persistent, and 2,4-DANT was again transformed to the 4-*N*-AcANT compound.

A potential pathway for the mineralization of 2,4,6-trinitrotoluene (TNT) was reported by Naumova et al., who studied TNT conversion by *Pseudomonas fluorescens* under aerobic and nitrate respiration conditions (18). Transformation of TNT was slower with aerobic cultures, and aminodinitrotoluenes (ADNTs) were the major metabolites observed. With nitrate respiration cultures, the rate of TNT transformation was accelerated, and the major metabolite observed was 2,4-diamino-6-nitrotoluene (2,4-DANT). Further conversion of 2,4-DANT was not significant under nitrate respiration conditions, but when *Pseudomonas fluorescens* was grown under aerobic conditions with 2,4-DANT as the sole source of nitrogen, 75% of the 2,4-DANT was degraded. Phloroglucinol and pyrogallol were reported as products of 2,4-DANT degradation (18, 19), indicating that all nitrogen had been removed from the aromatic ring. Separately, Walker and Taylor (33) reported the conversion of phloroglucinol to pyrogallol by a *Fusarium solani* strain; pyrogallol was then metabolized via cleavage of the aromatic ring (*meta*-fission pathway). This information suggested that further degradation of 2,4-DANT leading to ring cleavage is feasible and that mineralization of TNT may be possible via the pathway shown in Fig. 1.

The original purpose of this study was to confirm the two-step bioremediation process for TNT suggested by Naumova et al. (18). In the first step, TNT would be reduced to 2,4-DANT under nitrate-reducing conditions, and in the second step, 2,4-DANT would be further transformed (nitrogen removed from the ring followed by ring cleavage) under aerobic conditions. The objectives were to determine (i) whether the pathway shown in Fig. 1 is valid for our organism (*Pseudomonas fluorescens*) and culture conditions and (ii) how much of the TNT transformed can be accounted for as metabolites in this pathway. While we were able to confirm the first step of this process, the second step was not observed. Rather, 2,4-DANT was transformed to a novel metabolite, 4-*N*-acetylamino-2-amino-6-nitrotoluene (4-*N*-AcANT). Here we report the experiments that led to the discovery of this novel metabolite.

MATERIALS AND METHODS

Organism. The *Pseudomonas fluorescens* strain used in all experiments was isolated from TNT-contaminated soils by Pat Unkefer's group at Los Alamos National Laboratories.

Medium. The medium was the same as Naumova's medium 3 (aerobic experiments) or medium 7 (anoxic experiments) (18) with the addition of the following trace minerals (grams per liter): $CuSO_4 \cdot 5H_2O$, 1.1×10^{-4}; $CoCl_2 \cdot 6H_2O$, 3.3×10^{-5}; H_3BO_3, 7.0×10^{-6}; $ZnCl_2$, 2.37×10^{-5}. Carbon, nitrogen, and nitroaromatic components varied; their concentrations are noted for each experiment.

Culture conditions. All cultures were incubated in 500-ml shake flasks at 150 rpm and room temperature (~25°C) unless noted otherwise. Continuous argon purge was used to exclude oxygen for anoxic experiments. Isolated colonies from nutrient agar plates were used to inoculate 200 ml of liquid nutrient medium [5.0 g of yeast extract per liter, 0.6 g of $(NH_4)_2SO_4$ per liter]. This medium was incubated for 20 h at 30°C and 150 rpm and then used to inoculate starter cultures as described below.

For anoxic, nongrowth experiments, 200 ml of starter medium containing 5.0 g of yeast extract per liter and 0.6 g of $(NH_4)_2SO_4$ per liter was inoculated with 2 ml of nutrient medium. Optical density, dry weight, and bicinchoninic acid (BCA) protein concentration values after 9 h of aerobic incubation were 1.070 (at 560 nm), 840 mg/liter, and 290 mg/liter, respectively. At this point, the entire starter culture was centrifuged and decanted. The recovered cell pellet was then used to inoculate 200 ml of the degradation medium (2.0 g of ethanol per liter, 1.0 g of KNO_3 per liter, approx. 100 mg of TNT per liter or 20 mg of 2-amino-4, 6-dinitrotoluene [2-ADNT] per liter).

Aerobic medium was inoculated from starter cultures containing the same carbon substrate that was to be used in the degradation experiment (2.0 g of ethanol or sodium acetate per liter) and 0.2 g of KNO_3 per liter (no nitroaromatic compounds). The 200-ml aerobic starter cultures were inoculated with 2 ml of nutrient medium and incubated for 24 h. Values for the optical density, dry weight, and BCA protein at this point were 0.350 (at 560 nm), 350 mg/liter, and 80 mg/liter, respectively. A 20-ml aliquot of this starter culture was then used to inoculate 200 ml of the degradation medium (2.0 g of ethanol or sodium acetate per liter, 0.0 or 0.2 g of KNO_3 per liter, 20 to 125 mg of 2,4-DANT per liter or 30 to 40 mg of 2,6-diamino-4-nitrotoluene [2,6-DANT] per liter).

Analytical. TNT and its metabolites were detected and quantified by high-pressure liquid chromatography (HPLC). The reversed-phase column was a Rainin Microsorb C8 80-315-C5 column. Separations were performed by using a solvent gradient program with 0.003 N H_2SO_4 (A) and acetonitrile (B) as the mobile phases. The gradient program was as follows: 0 to 4 min, 90% A–10% B; 5 to 15 min, 50% A–50% B; 16 to 25 min, 15% A–85% B; 26 to 30 min, 90% A–10% B. Changes between compositions were made via a linear ramp over 1 min. The total flow rate was held constant at 1.0 ml/min. Compounds were detected by measuring UV A_{254} with a Waters 486 tunable absorbance detector. Selected samples were run on a separate HPLC system with a Beckman module 168 diode array detector. 2-Hydroxylamino-4,6-dinitrotoluene (2-HADNT), 4-hydroxylamino-2,6-dinitrotoluene (4-HADNT), 2-ADNT, 4-amino-2,6-dinitrotoluene (4-ADNT), 2,4-DANT, 2,6-DANT,4,4′,6,6′-tetranitro-2,2′-azoxytoluene (2,2′-AZ), 2,2′,6,6′-tetranitro-4,4′-azoxytoluene (4,4′-AZ), and 4-*N*-AcANT were identified by HPLC comparisons with standards. Ethanol was also quantified via HPLC; the isocratic method involved a Bio-Rad HPX-87H column at 65°C, a flow rate of 0.6 ml of 0.008 N H_2SO_4 per min for 22 min, and a refractive index detector. 4-*N*-AcANT was identified by comparison with a standard by both HPLC (diode array scan) and gas chromatography-mass spectrometry analysis. The identity of the standard was verified via nuclear magnetic resonance spectroscopy (by Ron Spanggord, SRI International, Menlo Park, Calif.). For gas chromatography-mass spectrometry analysis, 4-*N*-AcANT was extracted from a culture sample with an equal volume of ethyl acetate. Gas chromatography-mass spectrometry analysis was performed on a Hewlett-Packard 5890 II gas chromatograph equipped with a Hewlett-Packard model 5971A

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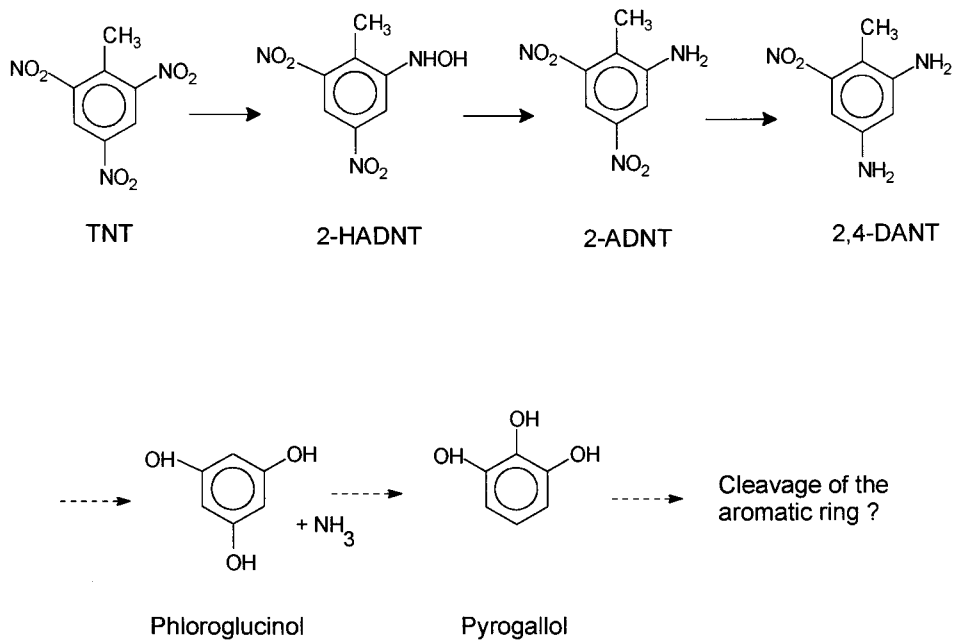

FIG. 1. Possible TNT biodegradation pathway for *Pseudomonas fluorescens* as suggested by Naumova et al. (18). Solid arrows indicate anoxic (nitrate-reducing) steps that were confirmed by our experiments. Dashed arrows show aerobic reactions that were not observed in our study.

mass-selective detector. The capillary column was an HP-5 column (cross-linked 5% phenylmethyl silicone; 50 m by 0.2 mm by 0.33 μm [film thickness]). Helium was the carrier gas. The oven temperature was maintained at 50°C for 2 min and then ramped to 77°C at 50°C/min. After 2 min at 77°C, the temperature was ramped at 25°C/min to a final temperature of 240°C. The 4-*N*-AcANT compound eluted at 24.5 min; prominent ions in the mass spectrum were *m/z* 43, 121, 150, 192, and 209 (parent ion). 4-*N*-AcANT elutes at 9.0 min in the HPLC analysis, and the diode array scan has one maximum at 220 nm with a shoulder at 245 nm and a shallow tail from 280 to 440 nm.

Nitrate analysis was performed colorimetrically with Szechrome NAS reagent (Polysciences, Inc.) by the method described in the product literature (20). For protein analysis, culture samples were centrifuged and decanted; the recovered pellet was then frozen, thawed, and dissolved in 0.1 N NaOH. This solution was then assayed for protein content by the BCA method (room temperature protocol) (25). Protein standards were prepared from bovine serum albumin.

Chemicals. TNT was obtained from Chem Service, Inc. 2,6-DANT, phloroglucinol, and pyrogallol were obtained from Aldrich. 2-ADNT, 4-ADNT, 2,4-DANT, 2,4,6-triaminotoluene (TAT), 4,4′-AZ, 2,2′-AZ, and 4-*N*-AcANT standards were generously provided by Ron Spanggord.

RESULTS

TNT degradation under anoxic (nitrate-reducing) conditions. Figure 2 shows TNT degradation under nongrowth, nitrate-reducing conditions. In this case, the cell inoculum was so large that population growth did not occur, as indicated by the flat protein curve. The nitrate concentration dropped from an initial value of 500 mg of NO_3^- per liter to less than 10 mg/liter at 38 h, and the ethanol concentration dropped from 1.77 g/liter at 0 h to 0.91 g/liter at 427 h. At 24 h, 98% of the initial TNT had disappeared, and at 52 h, the amount of ADNTs (2-ADNT/4-ADNT) present accounted for 42% of the TNT present initially. HADNT isomers (2-HADNT and 4-HADNT) and azoxy compounds (2,2′-AZ and 4,4′-AZ) were prominent at 24 h but had disappeared by 111 and 64 h, respectively. These compounds most probably account for "missing TNT" before 111 h; quantitative standards were not available for these compounds.

The decline in ADNT concentration after 52 h was accompanied by increasing concentrations of 2,4-DANT and 2,6-DANT. The 2,6-DANT that formed remained persistent for the duration of the experiment, while the 2,4-DANT concentration declined after 186 h. This decline was associated with the accumulation of an initially unknown compound that has now been identified as 4-*N*-AcANT (Fig. 3). Final 2,6-DANT, 2,4-DANT, and 4-*N*-AcANT concentrations at 427 h account for 11, 6, and 67%, respectively, of the TNT degraded.

The large error bars for 2,4-DANT and 4-*N*-AcANT concentrations between 186 and 376 h (Fig. 2A) are due to different 2,4-DANT–to–4-*N*-AcANT conversion rates among the three replicates. At 234 h, 2,4-DANT and 4-*N*-AcANT concentrations were 0.022 and 0.277 mM, respectively, in one flask and 0.267 and 0.064 mM, respectively, in another. However, the total TNT material balance (amount of degraded TNT present as known metabolites) for any individual culture was no less than 80% during the period between 186 and 376 h. Further reduction of 2,4-DANT or 2,6-DANT to form TAT was not observed. Unknown aromatic metabolites were observed at 10.4, 11.0, and 11.6 min in the HPLC analysis; the sum of these unknown metabolite peak areas (at 254 nm) at 427 h equaled 12% of the TNT peak area in the initial sample (0 h).

Three control experiments were run for the experiment shown in Fig. 2. In the first, 2-ADNT rather than TNT was used as the nitroaromatic substrate. This experiment confirmed the pathway observed in Fig. 2, namely, the reduction of 2-ADNT to 2,4-DANT and 2,6-DANT followed by the conversion of 2,4-DANT to 4-*N*-AcANT. Essentially 100% of the 2-ADNT initially present was accounted for as 2,4-DANT and 2,6-DANT at one point, and a decline in 2,4-DANT concentration at the end was accompanied by the formation of the 4-*N*-AcANT compound. The second control was run without any nitrate present to determine whether TNT reduction was associated with nitrate reduction. In this case, the same pathway was observed, but the transformations occurred at a significantly higher rate; 99% of the TNT initially present had disappeared by 8.5 h. TNT reduction was not observed in a third control in which no inoculum was added. In this experiment, 90% of the initial TNT was still present after 355 h.

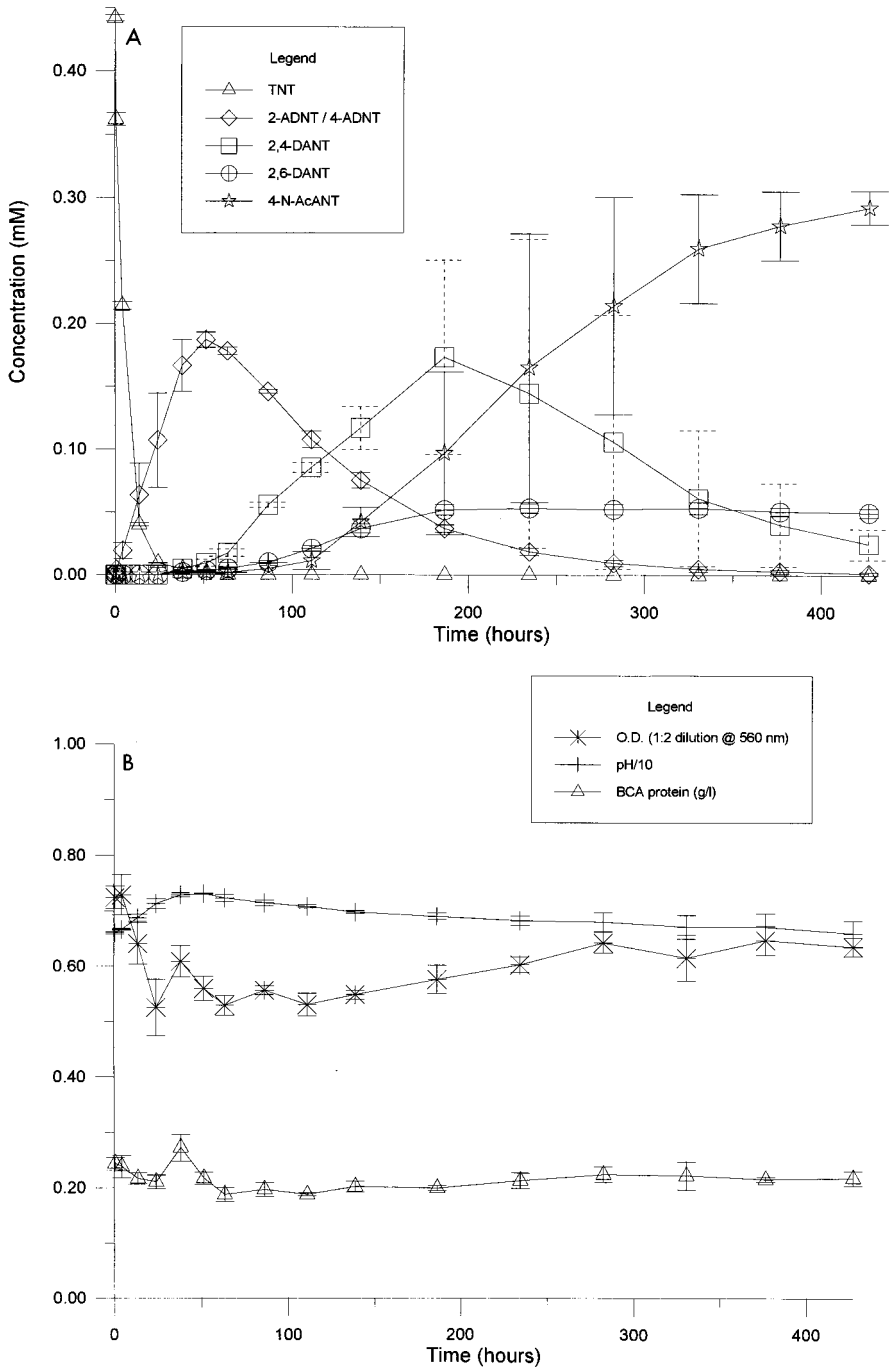

FIG. 2. TNT transformation by *Pseudomonas fluorescens* under anoxic (nitrate-reducing), nongrowth conditions. Triplicate cultures contained 1.77 g of ethanol per liter, 0.5 g of NO_3^- per liter, and 100 mg of TNT per liter initially. Error bars are 1 standard deviation. (A) Concentration of TNT and resulting metabolites. Wide, solid error bars are for 4-*N*-AcANT; wide, dashed error bars are for 2,4-DANT. (B) Optical density (O.D.), pH, and BCA protein values.

Further degradation of 2,4-DANT and 2,6-DANT. Since reduction of TNT to 2,4-DANT and 2,6-DANT was observed under nitrate-reducing conditions, the obvious next step was to investigate further degradation of these compounds. Naumova et al. (18) indicated that aerobic, nitrogen-limited conditions would promote removal of nitrogen from the aromatic ring and perhaps lead to ring cleavage (Fig. 1). Figure 4 shows the results of our attempt to duplicate their findings. In this experiment, ethanol was the carbon source and 2,4-DANT was the sole source of nitrogen. The amount of 2,4-DANT present initially (50 mg/liter) constituted 12% of the estimated nitrogen required to utilize all the ethanol. A fermentation with ethanol but no 2,4-DANT or other nitrogen source served as a control. Culture growth as evidenced by a slight increase in optical density between 0 and 50 h was due to a residual amount of nitrogen in the inoculum; the same increase was also observed in the no-nitrogen control. The protein concentration remained essentially constant for the duration of the

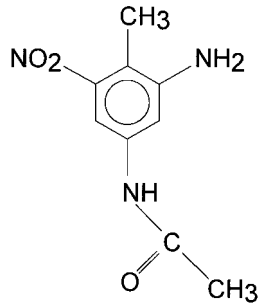

FIG. 3. Structure of 4-*N*-AcANT.

experiment, indicating that no significant culture growth had occurred. The ethanol concentration dropped from 1.8 g/liter initially to 1.3 g/liter at 409 h.

The disappearance of 2,4-DANT (Fig. 4) was accompanied by the formation of 4-*N*-AcANT. At 409 h, 78% of the 2,4-DANT initially present had been transformed; 88% of the 2,4-DANT that disappeared was accounted for as 4-*N*-AcANT. Similar experiments were performed with 2,4-DANT as the sole nitrogen source and sodium acetate as the carbon source. Once again, the 2,4-DANT was merely transformed to the 4-*N*-AcANT compound (91% of the 2,4-DANT transformed was accounted for as 4-*N*-AcANT). No 2,4-DANT degradation/transformation was observed in an aerobic, uninoculated control flask after 360 h; hence, the conversion to 4-*N*-AcANT was shown to be biological.

Conversion of 2,4-DANT to 4-*N*-AcANT was also observed in aerobic cultures that were supplemented with 0.2 g of KNO_3 per liter (in addition to 2,4-DANT and sodium acetate or ethanol). Significant growth as indicated by optical density measurement was observed, and the acetylation of 2,4-DANT was associated with this growth phase. In these experiments, 2,4-DANT acetylation stopped after the optical density peaked.

Attempts to further degrade 2,6-DANT were made under a variety of culture conditions: anoxic, aerobic with limited KNO_3 present, and aerobic with 2,6-DANT as the sole source of nitrogen. Either sodium acetate or ethanol was supplied as a carbon source. Culture growth occurred only when nitrate was provided, and no 2,6-DANT degradation was observed in any of the experiments.

DISCUSSION

The bioreduction of TNT to 2,4-DANT has been observed under aerobic conditions (15, 18, 23) but occurs more readily in an anoxic or anaerobic environment (21). TNT reduction is based on gratuitous transformation activities (22) and is not necessarily linked to inorganic nitrate reduction (1), as evidenced by our no-nitrate control experiment. While no significant TNT mineralization occurred in our anoxic experiments, the reduction of aromatic nitro groups to amino groups does lower the toxicity (34) and the mutagenicity (30, 34) of the compound. The fact that up to 90% of the TNT degraded was accounted for as known metabolites (2,4-DANT, 2,6-DANT, and 4-*N*-AcANT) indicates that this reductive pathway has the potential for high degradation efficiency. It also provides a means of degrading 2-ADNT/4-ADNT, compounds that are commonly observed (2–4, 8, 11, 15, 18, 23, 32, 35) and often found to be persistent (11, 22, 35) under aerobic conditions. Unfortunately, the final metabolites observed in our anoxic experiments appear to be dead ends, because both 4-*N*-AcANT and 2,6-DANT were persistent in all of our experiments. The production of the 2,6-DANT isomer is unusual, because 2,4-DANT is typically the only diaminonitrotoluene isomer observed (21). The reduction of 2,4-DANT or 2,6-DANT to TAT was not observed in our experiments, consistent with reports that this reaction occurs only with strictly anaerobic strains (10, 21, 22). Complete reduction of TNT to TAT under strictly anaerobic conditions may be the key to further degradation and eventual mineralization. Crawford has isolated an anaerobic consortium that produces acetate as a nonaromatic TNT degradation product (7). He speculates that TAT is part of this pathway.

Attempts to further degrade 2,4-DANT under aerobic, nitrogen-limited conditions were unsuccessful. 2,4-DANT was merely transformed to the same 4-*N*-AcANT compound produced in the anoxic cultures, and growth as indicated by BCA protein concentration was not observed (Fig. 4). This is in contrast with the results of Naumova et al. (18), who observed growth of a *Pseudomonas fluorescens* species with ethanol as a carbon source and 2,4-DANT as the sole nitrogen source. They report that 2,4-DANT was converted to phloroglucinol and pyrogallol, but neither of these substances was observed (HPLC analysis) in our 2,4-DANT cultures. While the amount of nitrogen present as 2,4-DANT in our experiments (12% of the theoretical value for Fig. 4) may not have been enough to provide significant growth, the conversion of 2,4-DANT to 4-*N*-AcANT does not result in a loss of nitrogen from the aromatic ring. Since 88% of the 2,4-DANT that disappeared was accounted for as 4-*N*-AcANT, we can conclude that 2,4-DANT did not serve as a significant nitrogen source for growth.

The only differences between our 2,4-DANT degradation medium and that of Naumova et al. (18) were the 2,4-DANT concentration (500 mg/liter versus a maximum of 125 mg/liter in our experiments) and the use of additional trace minerals in our medium (see Materials and Methods). Our inoculation procedure may also have been different; a description of their inoculation procedure was not included in their paper. Since we used a different strain of *Pseudomonas fluorescens*, metabolic differences between our strain and theirs may account for the different results. However, the biotransformation of 2,4-DANT to phloroglucinol appears questionable at this point, especially since this would most probably require a number of intermediate steps that have not been identified.

Schackmann and Müller (23) observed *N*-acetyl compounds in the aerobic conversion of various nitroaromatic compounds by resting cells of a *Pseudomonas* sp. However, in the case of TNT, they observed ADNTs and DANTs but did not report any *N*-acetyl compounds as metabolites. We do not have any definitive explanations why the cells convert 2,4-DANT to 4-*N*-AcANT; one possibility is that the 4-*N*-AcANT compound is somehow less toxic to the cells and the reaction serves as a detoxification mechanism. The reaction of 2,4-DANT with acetate involves a nucleophilic attack of an amine group on a carbonyl carbon; the resulting 4-*N*-AcANT compound (Fig. 3) is less basic and perhaps less toxic. The acetylation of aniline derivatives by soil microorganisms does serve to detoxify such compounds (31). Aminoglycoside antibiotic resistance for numerous bacterial species including *Pseudomonas* species is accomplished via N-acetylation (5). *Salmonella typhimurium* strains possessing high levels of arylamine *N*-acetyltransferase catalyze the N-acetylation of the aromatic amine benzidine (24). Thus, the ability to N-acetylate amine compounds is not uncommon in bacteria. The N-acetylation of amine compounds is reported to be acetyl coenzyme A (acetyl-CoA) dependent (24, 29, 36). This acetyl-CoA dependence may explain why in aerobic experiments with cultures supplemented

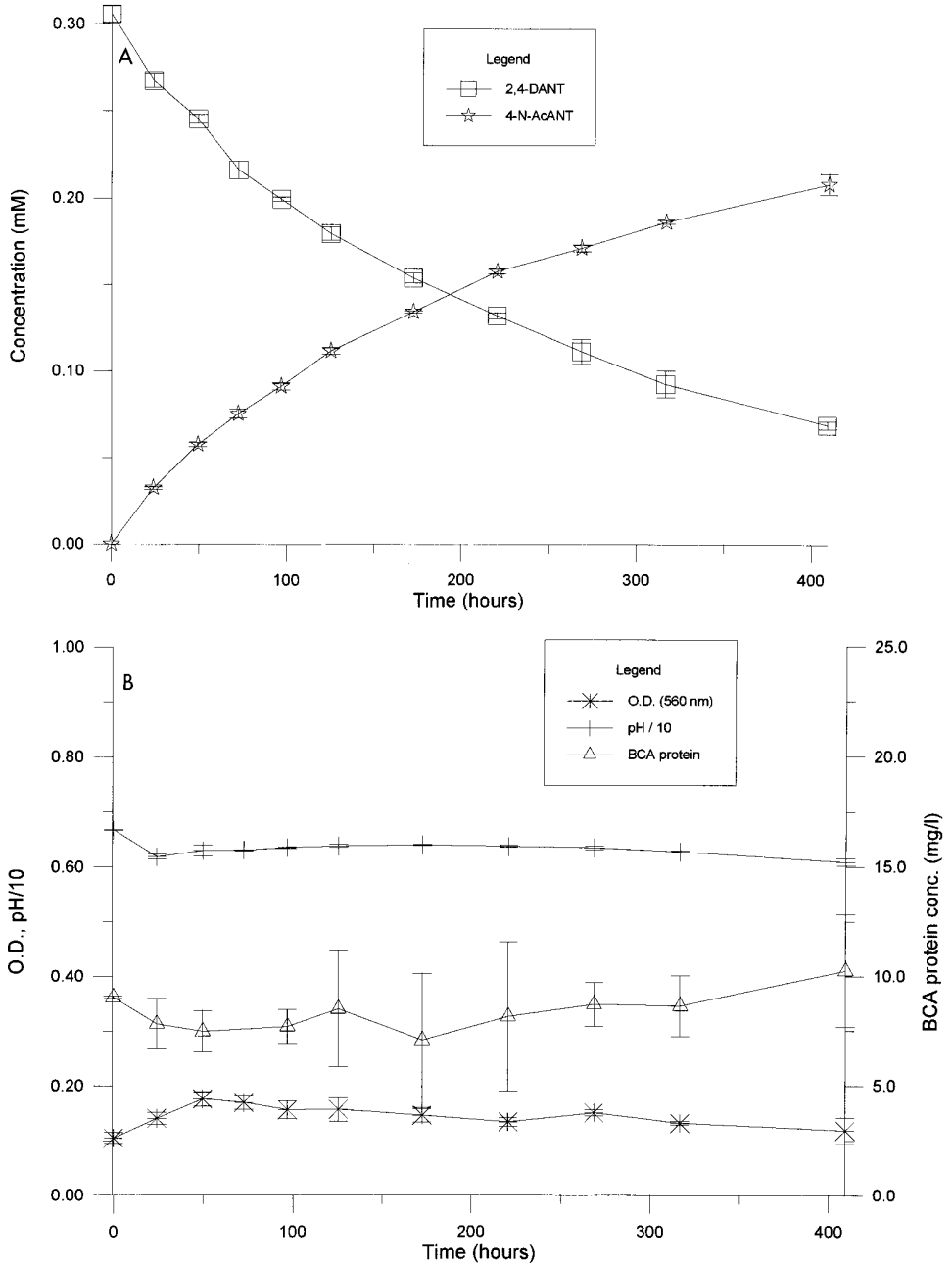

FIG. 4. 2,4-DANT transformation by *Pseudomonas fluorescens* under aerobic conditions. Duplicate cultures contained 1.8 g of ethanol per liter and 50 mg of 2,4-DANT per liter (sole nitrogen source) initially. (A) Conversion of 2,4-DANT to 4-*N*-AcANT. (B) Optical density (O.D.), pH, and BCA protein values. Error bars are 1 standard deviation.

with KNO_3, the N-acetylation of 2,4-DANT stopped at the same time that culture growth ceased. In this case, exhaustion of the growth substrate may have also depleted the acetyl-CoA pool.

The N-acetylation of 2-ADNT/4-ADNT could not have been a significant reaction in the anoxic cultures in which 2,4-DANT acetylation took place, since 84% of the TNT degraded was accounted for as known metabolites (Fig. 2). The lower basicities of 2-ADNT/4-ADNT than of 2,4-DANT may have prevented their N-acetylation, since the ADNT amine group is less nucleophilic. Glover et al. (12) report pK_a values for 2-ADNT, 4-ADNT, and 2,4-DANT as 0.59, 1.23, and 3.54, respectively. Acetylation of the 2,6-DANT isomer was also not observed in our experiments. In this case, steric hindrances from the *ortho* methyl group may have prevented the acetylation of the amine groups. The same steric effect was observed in the degradation of 2,4-dinitrotoluene by *Mucrosporium* spp. (14). Both aminonitrotoluene isomers were observed as reduction products, but only 4-amino-2-nitrotoluene was acetylated.

The persistence of 4-*N*-AcANT in our experiments suggests that the acetylation of 2,4-DANT is not a useful reaction in terms of bioremediation. However, the N-acetylation of aniline by sewage sludge is reversible and does not interfere with its ultimate mineralization (13). The formylated derivative of 2,4-

DANT, 4-*N*-formylamino-2-amino-6-nitrotoluene, is a lignin peroxidase substrate and is rapidly degraded by ligninolytic cultures of *Phanerochaete chrysosporium* (17). This suggests that such cultures may be capable of degrading the acetylated derivative of 2,4-DANT (4-*N*-AcANT) produced in our experiments. *P. chrysosporium* cultures are known to mineralize ADNTs (2-ADNT/4-ADNT) (16) and may be able to mineralize 2,4-DANT as well (17). Thus, all the products observed in our anoxic TNT cultures are known or potential substrates for *P. chrysosporium.*

P. chrysosporium is capable of mineralizing TNT directly (6, 9, 16, 26, 27), but the rates are inhibited at higher concentrations (16, 26). ADNT mineralization rates are not adversely affected at higher concentrations (16), and ADNT is less toxic to *P. chrysosporium* than is TNT (28). Thus, it might be advantageous to first reduce TNT to ADNT with a bacterial species in a separate reactor. A potential system for the treatment of TNT-contaminated water would consist of an anoxic reactor for the reduction of TNT by *Pseudomonas fluorescens* followed by the mineralization of reduced metabolites in a second, aerobic reactor containing *P. chrysosporium*. This system would avoid the problem of TNT inhibition in fungal cultures while providing a means of degrading the reduced metabolites observed in our study.

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