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TNT biotransformation and detoxification by a *Pseudomonas aeruginosa* strain

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Abstract

Successful microbial-mediated remediation requires transformation pathways that maximize metabolism and minimize the accumulation of toxic products. *Pseudomonas aeruginosa* strain MX, isolated from munitions-contaminated soil, degraded 100 mg TNT L^{-1} in culture medium within 10 h under aerobic conditions. The major TNT products were 2-amino-4,6-dinitrotoluene (2ADNT, primarily in the supernatant) and 2,2′-azoxytoluene (2,2′AZT, primarily in the cell fraction), which accumulated as major products via the intermediate 2-hydroxylamino-4,6-dinitrotoluene (2HADNT). The 2HADNT and 2,2′AZT were relatively less toxic to the strain than TNT and 2ADNT. Aminodinitrotoluene (ADNT) production increased when yeast extract was added to the medium. While TNT transformation rate was not affected by pH, more HADNTs accumulated at pH 5.0 than at pH 8.0 and AZTs did not accumulate at the lower pH. The appearance of 2,6-diamino-4-nitrotoluene (2,6DANT) and 2,4-diamino-6-nitrotoluene (2,4DANT); dinitrotoluene (DNT) and nitrotoluene (NT); and 3,5-dinitroaniline (3,5DNA) indicated various routes of TNT metabolism and detoxification by *P. aeruginosa* strain MX.

Introduction

Contamination of soil and water from 2,4,6-trinitrotoluene (TNT) has occurred during the disposal of wastewater from munitions production, loading and packing operations in many countries. The persistence of TNT and its metabolites are of environmental concern because they may be toxic to fish (Osmon & Klausmeier 1972; Smock et al. 1976), algae (Smock et al. 1976; Won et al. 1976; Bennett 1994), microorganisms (Klausmeier et al. 1973; Won et al. 1976; Rieger & Knackmuss 1995), higher plants (Palazzo & Leggett, 1986) and humans (Yinon 1990). Research has shown that the major products of aerobic TNT biotransformation by bacteria are 4-amino-2,6-dinitrotoluene (4ADNT), 2-amino-4,6-dinitrotoluene (2ADNT), and various azoxytoluene (AZT) condensation products from hydroxylaminodinitrotoluenes (HADNTs) and nitrosodinitrotoluenes (NoDNTs) (Kaplan & Kaplan 1982; Schackmann & Mueller 1991; Bumpus & Tatarko 1994; Alvarez et al. 1995; Bradley et al. 1995; Drzyzga et al. 1998; Esteve-Núñez & Ramos 1998; Kim & Song 2000; Esteve-Núñez et al. 2001). Diaminonitrotoluene (DANT) also has been identified (Won et al. 1974; Funk et al. 1993; Daniel et al. 1995; Fiorella & Spain 1997; Drzyzga et al. 1998; Esteve-Núñez & Ramos 1998), but complete reduction of TNT to triaminotoluene (TAT) required strict anaerobic conditions (Ederer et al. 1997; Drzyzga et al. 1998) and has not been observed in aerobic soils. Haidour & Ramos (1996) and Martin et al. (1997) revealed the capacity of some *Pseudomonas* strains to aerobically denitrate TNT, producing 2,4-dinitrotoluene (2,4DNT) and 2,6-dinitrotoluene (2,6DNT).

Although Hankenson & Schaeffer (1991) reported that TNT was 20- to 50-fold more toxic than a mixture of DANT and ADNT, toxicity may limit microbial degradation of TNT at highly contaminated sites. Research conducted in our laboratory indicated that *Pseudomonas savastanoi* rapidly transformed TNT to 2ADNT, 4ADNT, and 2,4DNT (Martin et al. 1997). While growth was sustained from the mid-log to stationary phases, a decline in cell population after 24 h may be due in part to accumulation of toxic metabolites. Subsequent research indicated 2-hydroxylamino-4,6-dinitrotoluene (2HADNT), 2ADNT, and 4,4′,6,6′-tetranitro-2,2′-azoxytoluene (2,2′AZT) were the major metabolites of aerobic TNT transformation by *P. aeruginosa* strain MX isolated from TNT-contaminated soil (Vasilyeva et al. 2000). Our present objective was an in-depth study of aerobic biotransformation and detoxification of TNT by the *P. aeruginosa* strain.

Materials and methods

Microorganism and chemicals

The isolate, *Pseudomonas aeruginosa* strain MX, was obtained from munitions-contaminated soil (Vasilyeva et al. 2000). Technical grade TNT was obtained from the Fort Detrick U.S. Biomedical Research and Development Laboratory (Frederick, MD). Carbon-14-ring-labeled TNT (137 MBq $mmol^{-1}$, radiochemical purity 98%) was custom-synthesized by NEN Research Products (Boston, MA). Analytical standards of 2ADNT, 4ADNT, 2HADNT, 4HADNT, 2,2′AZT, 2′,4AZT, 4,4′AZT, and 2,6DA4NT, 2,4DA6NT and 2,4,6-trihydroxytoluene (THT) were provided by R. Spanggord (SRI International, Palo Alto, CA). Cyclohexanol, 2,4DNT, 2-amino-4-nitrotoluene (2A4NT), and 4-nitrotoluene (4NT) were obtained from Aldrich (Milwaukee, WI). Cyclohexanone, 3,5-dinitroaniline (3,5DNA), yeast extract (YE), and salts for preparing culture media and Tryptic Soy Agar (TSA) were purchased from Sigma Chemical Co. (St. Louis, MO). TAT was obtained from Chem Service (West Chester, PA). All organic solvents were HPLC grade.

Culture conditions and growth measurements

P. aeruginosa strain MX was grown in culture medium containing the following (g L^{-1} distilled water): $MgSO_4$ (0.1), K_2HPO_4 (3.5), KH_2PO_4 (1.5), TNT (0.1) and yeast extract (1.0). An EDTA trace element solution (1.0 mL) was added to the culture medium, as described by Alef & Nannipieri (1995). Culture flasks were incubated on an orbital shaker (150 strokes per min) at 28 °C under aerobic conditions. Turbidity of the cultures was monitored at 560 nm using a UV-VIS scanning spectrophotometer (Model UV-2101PC, Shimadzu, Japan). Control cultures were grown without TNT or with no inoculation under the same conditions.

TNT transformation

TNT transformation was determined in culture medium containing 100 mg TNT L^{-1} and inoculated with *P. aeruginosa* strain MX. During the incubation, 1 mL samples were withdrawn for analysis and the cells were harvested by centrifuging for 10 min at $14,000 \times g$. The cell pellets were extracted by vortexing three times for 1 min with 1 mL acetonitrile and centrifuging again. TNT and its major metabolites (ADNTs, HADNTs, and AZTs) were determined by HPLC and changes in concentration were monitored. To detect 2,6DANT and cyclohexanone, strain MX was grown for 3 days as described above. After centrifugation, 500 mL of supernatant was extracted three times with 100 mL of methylene chloride. The extract was concentrated by rotary evaporation, H_2O removed with sodium sulfate (Na_2SO_4), and 3 μL samples were analyzed by GC/MS. Controls without TNT were included in the analyses.

Culture medium components and their concentrations were modified in the subcultures. TNT concentrations were 20, 40, 60, 80, and 100 mg L^{-1} and yeast extract concentration varied from 0.2 to 1.0 g L^{-1}. The pH of the culture medium varied from 5.0 to 8.0.

Mass balance of ^{14}C-TNT

Carbon utilization was tracked by adding ^{14}C-TNT to 30 mL of culture medium (50,000 dpm mL^{-1}). After incubating for 3 d, the culture medium was centrifuged and the supernatant was extracted three times with 30 mL of methylene chloride. After acidifying to pH 1.0 with HCl, the aqueous phase was extracted with ethyl acetate as described above. The centrifuged cell fraction (pellet) was extracted by vortexing and centrifuging three times with 10 mL acetonitrile. Total ^{14}C in the extracts and in the remaining aqueous solution was determined by mixing 0.5 mL of sample with 6 mL Ultima Gold Cocktail (Packard Instrument Company, Meriden, CT) and liquid scintillation counting (LSC, Packard 1900TR liquid scintillation

counter, Packard Instrument Co., Downers Grove, IL). Unextractable ^{14}C remaining in the pellet was determined by combusting to $^{14}CO_2$ in a biological oxidizer (Packard, Tri-Carb B306, Packard Instrument Co., Downers Grove, IL). The $^{14}CO_2$ was trapped in a mixture (3 : 2, v/v) of Carbosorb and Permaflour (Packard Instrument Company, Meriden, CT) and quantified by LSC. The ^{14}C-TNT and its ^{14}C-metabolites were quantified by HPLC using a radioactivity detector (Radiomatic, Series A-200, Packard Instrument Co., Downers Grove, IL).

Toxicity of TNT and major metabolites to P. aeruginosa *strain MX*

To determine the toxicity of TNT to strain MX, culture medium was incubated with various TNT degradation intermediates and products as previously described. During incubation, the bacteria were periodically enumerated by plating on TSA and counting the number of colony forming units (CFU) per mL. Solutions of TNT, 2HADNT, 2ADNT, 4ADNT, 2,4DANT, or 2,2′AZT in acetone (50 mg L^{-1}) were added to 250-mL Erlenmeyer flasks and the acetone was allowed to evaporate. Acetone was used to obtain a uniform distribution of fine particles in the flask after evaporation and thus promote maximum dissolution in the medium. One-day-old cultures were transferred to the flasks and incubated as previously described.

After 12, 24, and 36 h, the medium was centrifuged. The supernatant was analyzed for TNT and its metabolites by HPLC and the pellet was used for electron microscopy. Bacteria growing with cyclohexanol, cyclohexanone, 2,6DANT; 4NT; THT; 2A4NT; TAT; 2,4DNT; 3,5DNA; TNT; 2HADNT; 2ADNT; 4ADNT; 2,4DANT; and 2,2′AZT at 50 mg L^{-1} were enumerated by plating on TSA and counting CFUs per mL. Although not unequivocally confirmed as products in our experiments, cyclohexanol, THT, and TAT were included for toxicity comparison because they are potential additional products of TNT degradation. Control cultures were grown in the absence of TNT or its metabolites.

Electron microscopy

Samples were prepared for electron microscopy by creating a bacteria pellet through centrifugation. Samples were fixed in 3% (w/v) glutaraldehyde in 0.1 M phosphate buffer (pH 6.8) at room temperature for 1 h. After rinsing with the buffer, the samples were post-fixed in 1% (w/v) osmium tetroxide in phosphate buffer for 1 h before dehydration in an ethanol series (50, 75, 90, and 100% for 20 min in each step) and propylene oxide. Samples were left in 1 : 1 ratio of propylene oxide and Epon 812 (Electron Microscopy Sciences, Fort Washington, PA) mixture overnight at room temperature before embedding in pure Epon 812. Polymerization of Epon 812 blocks was performed at 60 °C for 1 d. After trimming the sample blocks, sections were cut with a LKB Ultrotome III equipped with a diamond knife. Sections mounted on 200-mesh copper grids were stained with lead citrate and uranyl acetate, and photographed with a transmission electron microscope (Philips 201, FEI Co., Hillsboro, OR) operated at 60 KeV.

Analytical methods

The cell culture supernatant was assayed for nitrite using a modified colorimetric method (Clesceri et al. 1989) and analyzed by HPLC. The centrifuged cell pellet was extracted with acetonitrile and analyzed by HPLC. Chromatographic analysis was conducted using a 250 × 4.6 mm Keystone Betasil NU column (Keystone Scientific Inc., Bellefonte, PA) with an isocratic mobile phase of acetonitrile and deionized water acidified with H_3PO_4 to pH 2.5 (55 : 45, v/v) at a flow rate of 1.0 mL min^{-1}. TNT and its metabolites were detected spectrophotometrically at 254 nm and quantified by comparison with pure analytical standards.

TNT transformation products were also confirmed by GC/MS (HP 6890 gas chromatograph equipped with a mass selective detector, EI mode) without derivatization. Separations were obtained using 5% cross-linked phenyl methyl siloxane capillary column (HP-5MS, 0.25 mm id by 30 m with a 0.25 μm film). The carrier gas was helium at 1 mL min^{-1}, the detector temperature was 280 °C, and the inlet temperature was 190 °C. The initial column oven temperature was held at 40 °C for 5 min, then increased 10 °C min^{-1} to 150 °C, held for 1 min, then increased 5 °C min^{-1} to 300 °C and held for 5 min.

Results

TNT biotransformation

Within 10 h of incubation with *Pseudomonas aeruginosa* strain MX, 100% of the TNT (100 mg L^{-1}) had disappeared from the culture medium and 2HADNT, 4HADNT, 2ADNT and 2,2′AZT were identified in the

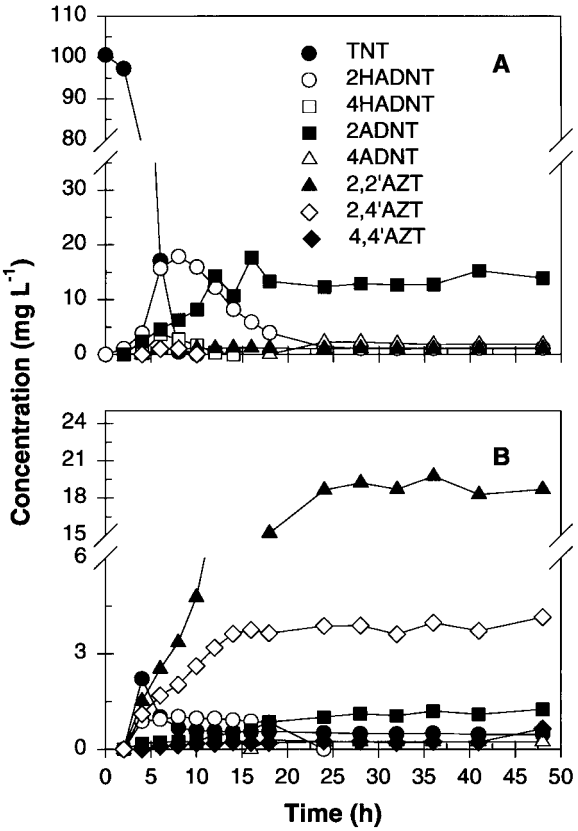

Figure 1. Metabolites of TNT degradation by *P. aeruginosa* strain MX found in the supernatant (A) and centrifuged cell fraction (B) from the culture medium.

supernatant (Figure 1A). The centrifuged cell fraction from the culture medium contained primarily 2,2′AZT, 2′,4AZT, 4,4′AZT and 2ADNT (Figure 1B). Cyclohexanone and 2,6DANT were also detected in methylene chloride extract by GC/MS. These metabolites were not detected in extracts of the control supernatant in the absence of TNT. Mass spectra from GC/MS analyses of cyclohexanone and 2,6DANT matched reference spectra. GC/MS analysis of the supernatant also revealed the presence of small amounts of 2,4DNT, 4NT, and 3,5DNA.

A mass balance for TNT transformation was obtained by growing *P. aeruginosa* strain MX in the presence of ^{14}C-TNT. After centrifuging the culture medium, 21.4% of the ^{14}C was found in the supernatant and 71.5% was in the cell pellet (Table 1). The greatest ^{14}C fraction (17%) in the supernatant was extracted by methylene chloride, which primarily contained 2ADNT (6.7%) and 2,2'AZT (7.7%). After acidification to pH < 1, approximately 3.3% of the ^{14}C was extracted by ethyl acetate and 1.1% remained in the aqueous fraction. Most of the ^{14}C associated with the centrifuged pellet was extracted by acetonitrile (70.3%). Metabolites identified in this solvent included 2,2′AZT (59.4%) and 2ADNT (1.3%), while 9.6% consisted of unidentified products. Approximately 0.9% of the ^{14}C in the pellet was extracted by 0.5 *N* NaOH and 0.3% of the ^{14}C was unextractable. Mineralization of ^{14}C-TNT to $^{14}CO_2$ was negligible (less than 2%). It is also possible that ^{14}C fractions comprising less than 2% of the total ^{14}C activity may be associated with impurities in the ^{14}C-TNT (98% radiochemical purity).

Growth of P. aeruginosa *strain MX with TNT and nitrite release*

TNT disappearance coincided with increased turbidity and nitrite concentration. Maximal microbial growth was observed at about 10 h of incubation with or without TNT (Figure 2A). Nitrite concentration in the culture medium increased after incubating for 3 h with *P. aeruginosa* strain MX and reached a maximum of about 18 μM NO_2^- after 12 h, which was sustained for more than 48 h (Figure 2B). Some nitrite was found in the uninoculated control containing TNT, but the concentration did not change with time. Increasing TNT concentration resulted in increasing nitrite (NO_2^-) in the culture fluid (data not shown), indicating that the strain was capable of eliminating —NO_2 from the TNT ring. Some nitrite was found in the uninoculated control containing 100 mg TNT L^{-1}, but the concentration remained nearly constant with time.

TNT transformation was limited at low yeast extract concentrations (Figure 3A). Cultures containing greater than 0.4 g yeast extract L^{-1} transformed greater than 99% of the TNT within 12 h of incubation. An increase in yeast extract concentration increased 2ADNT in the culture medium (Figure 3B), suggesting that the yeast extract was a major energy source for TNT transformation by *P. aeruginosa* strain MX.

Toxicity of TNT and its major metabolites

To determine the potential toxicity of TNT and its major metabolites, cultures of strain MX were grown in the presence of TNT. Plating on TSA indicated bacterial viability in the presence of TNT was not significantly different from the control for the first 12 h of incubation. However, viability decreased by about 70% after incubating with TNT for 32 h (Figure 4A). When added at the stationary growth phase (after

Table 1. Transformation of TNT after incubating *P. aeruginosa* strain MX in liquid culture medium with ^{14}C-TNT (0.1 g L^{-1}) and yeast extract (1 g L^{-1}) for 3 days

Metabolites	Supernatant			Centrifuged precipitate			Total
	CH_2Cl_2	E.A.[a]	Water	CH_3CN	NaOH	Combustion	
			(%)				
Azoxy toluenes	7.7	0.9	–	59.4	–	–	68.0
ADNTs	6.7	0.1	–	1.3	–	–	8.1
Other compounds	2.6	2.3	1.1	9.6	0.9	0.3	16.8
Unaccountable	–	–	–	–	–	–	7.1
Total	17.0	3.3	1.1	70.3	0.9	0.3	100.0

[a] Ethyl acetate.

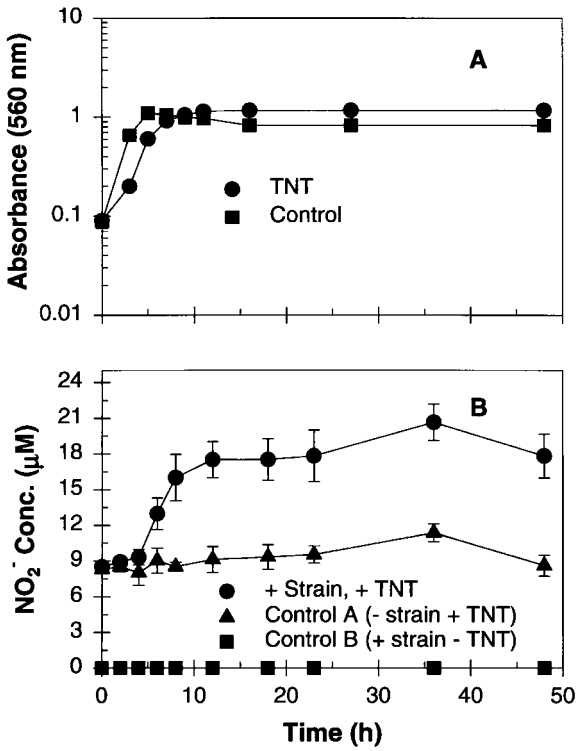

Figure 2. Change of turbidity of culture medium containing yeast extract (1.0 g L^{-1}) with TNT (60 mg L^{-1}) and without TNT inoculated by *P. aeruginosa* strain MX (A). Y axis is $\log_{10}$ scale. Nitrite concentration in the culture medium containing yeast extract (1.0 g L^{-1}) with TNT (60 mg L^{-1}) and without TNT after inoculation with *P. aeruginosa* strain MX (B).

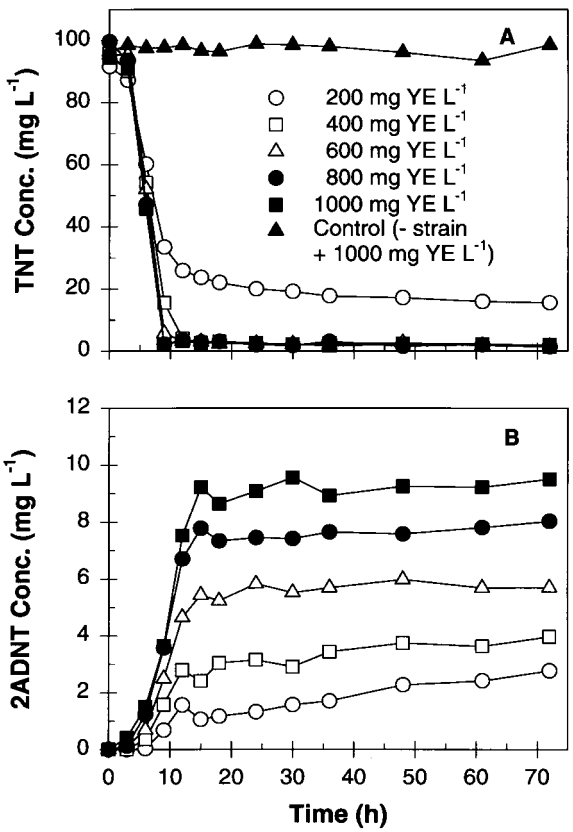

Figure 3. Influence of yeast extract concentration on TNT degradation rate (A) and 2ADNT production (B).

24 h of incubation), TNT, 2,4DNT and 3,5DNA were most toxic among the evaluated compounds, while cell viability in the presence of cyclohexanol was not different from the control. Although 2,6DANT, 4NT, 2,4DANT, 2,2′AZT and 2HADNT were relatively less toxic than TNT and 3,5DNA, these compounds significantly decreased cell viability compared to the control (Figure 4B). Interestingly, 3,5DNA and 2,4DNT were more toxic than the parent compound (TNT).

Separate experiments indicated the relative biodegradability of TNT compared to its transformation products (Table 2). After 36 h, about 37% of the TNT was transformed in the culture medium to ADNTs,

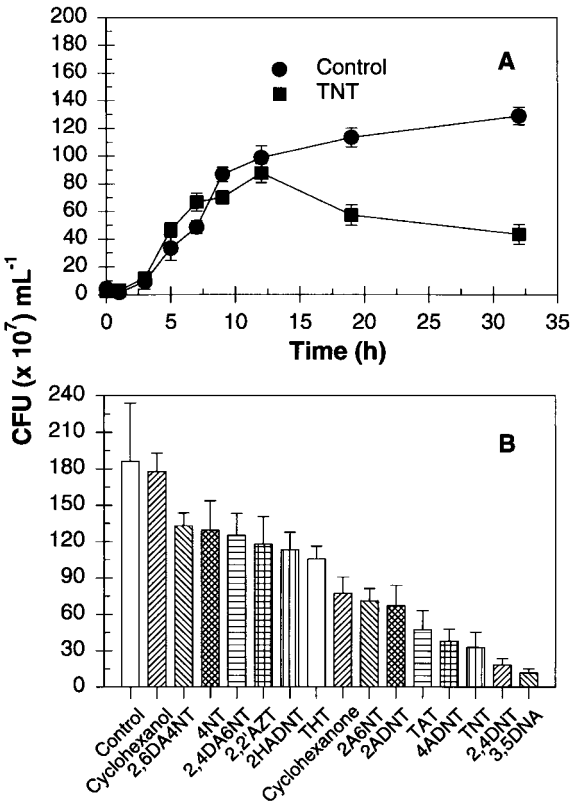

Figure 4. Bacterial counts (CFU, colony forming units) in the cultural medium containing yeast extract (1.0 g L^{-1}) with TNT (100 mg L^{-1}) and without TNT inoculated by *P. aeruginosa* strain MX after plating on tryptic soy agar (A). Bacterial counts (CFU, colony forming units) of *P. aeruginosa* strain MX in liquid culture medium containing yeast extract (1.0 g L^{-1}) with major TNT metabolites and without metabolites (control) after incubating for 36 h with stationary phase of growth (B).

DANTs, 2,2′AZT and an unidentified product (HPLC RT = 5.0 min). By comparison, 2ADNT was less readily degraded, with 7.2% transformed to 2,6DANT. The 2HADNT was completely transformed to 2ADNT, 2,6DANT, 2,2′AZT and the unidentified product at RT = 5.0 min.

Examining the bacteria cells with an electron microscope at stationary phase (12 h) in the absence of TNT or its metabolites showed the presence of healthy cells, containing well-defined cell walls, membranes, and cell contents (Figure 5A). In contrast, cultures incubated in the presence of TNT contained many cells exhibiting condensation of material in the cell centers. There also appeared to be some loss in the density of the bacterial cytoplasm (Figure 5B). Prolonged incubation of *P. aeruginosa* strain MX in culture media containing 50 mg of 2ADNT or 4ADNT L^{-1} resulted in markedly altered cell contents. *Pseudomonas aeruginosa* strain MX showed condensation of the DNA component and ultimately resulted in necrosis (Figure 5C and D).

Effect of pH

While the optimal pH range for TNT transformation was between 7.0 and 8.0, more 2HADNT (Figure 6A) and 2ADNT (Figure 6B) accumulated at pH 5.0 than at pH 8.0. The accumulation of 4HADNT was similarly affected by pH (data not shown). At lower pH, 2,2′AZT accumulated much more slowly in the cell fraction (centrifuged pellet) (Figure 6C).

Discussion

Pseudomonas aeruginosa strain MX, isolated from TNT-contaminated soil, completely transformed 100 mg TNT L^{-1} in the medium within 10 h. The major TNT transformation products were 2ADNT, which was present in the supernatant, and 2,2′AZT, primarily found in the centrifuged cell fraction. Other researchers have reported bacteria-mediated reduction of at least one TNT nitro group (Channon et al. 1944; Boopathy et al. 1993, 1994; Bradley et al. 1994; Haidour & Ramos 1996). The reduction intermediate, 2HADNT, was transiently found in the medium, prior to detecting 2ADNT. Detection of cyclohexanone was consistent with TNT transformation described by Chung who suggested that TNT may be transformed to 2ADNT and 4ADNT, and subsequently to 2,4DANT (e.g., by TNT reductase of Desulfovibrio spp.), which is then converted acetate via 2-nitrobenzoic acid, with cyclohexanone, 2-methylpentanoate, and butyrate as intermediates (Chung, Darling Marine Center, University of Maine, pers. comm.) (Figure 7).

In our experiments with ^{14}C-TNT, AZTs accounted for approximately 71.5% of the ^{14}C after complete transformation of the TNT, which is similar to previous investigations (Carpenter et al. 1978). While the condensation of HADNTs with NoDNTs forms AZTs (Won et al. 1974; McCormick et al. 1976; Kaplan & Kaplan 1982; Esteve-Núñez et al. 2001), AZT production decreases at lower pH (Funk et al. 1993). Khan et al. (1997) reported rapid transformation of hydroxylamines to undetermined end products without detecting AZTs at low pH (4.1–4.5), and similar results were obtained in our experiments. After 24 h, as much as ten times more 2,2′AZT was produced at pH

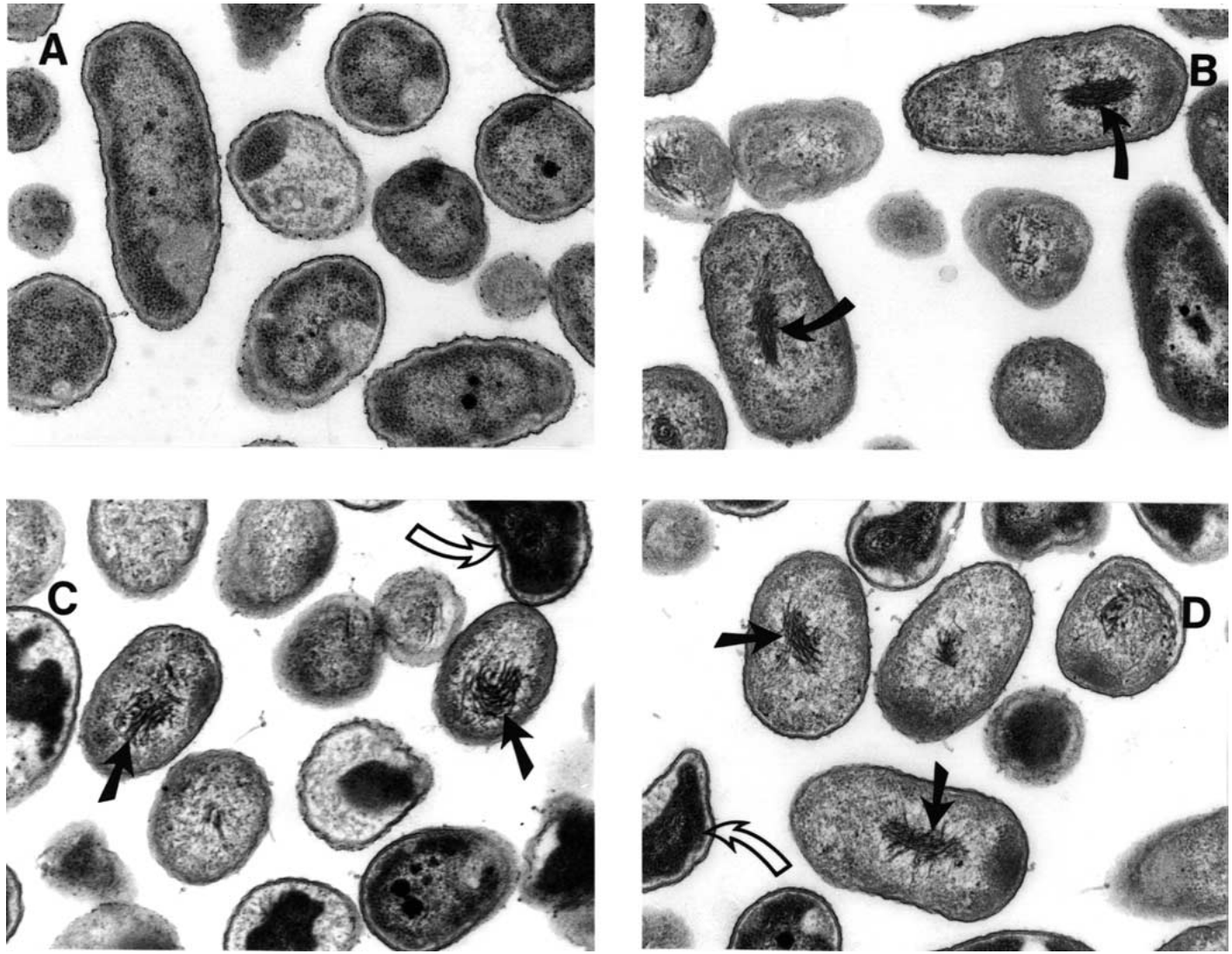

Figure 5. Transmission electron micrographs of *P. aeruginosa* strain MX in the cultural medium containing yeast extract without TNT (control) (A) and with TNT (B) for 12 h of incubation; 2ADNT (C); and 4ADNT (D) after incubating 24 h at the stationary growth phase. Solid arrows indicate condensation of the DNA component and blank arrows indicate necrotic cells.

Table 2. Transformation of TNT, 2HADNT, 2ADNT, 2,4DA6NT or 2,2′AZT (50 mg L^{-1}) in liquid culture medium containing yeast extract (1.0 g L^{-1}) after 36 h incubation with *P. aeruginosa* strain MX of stationary phase

Time (h)	Initial compound				
	TNT	2HADNT	2ADNT	2,4DA6NT	2,2′AZT
			(%)		
0	100	100	100	100	100
36	63.1 (36.9)[a]	0 (100)	92.8 (7.2)	82.1 (17.9)	74.9 (25.1)

[a]Paraenthetic values indicate transformation (%).

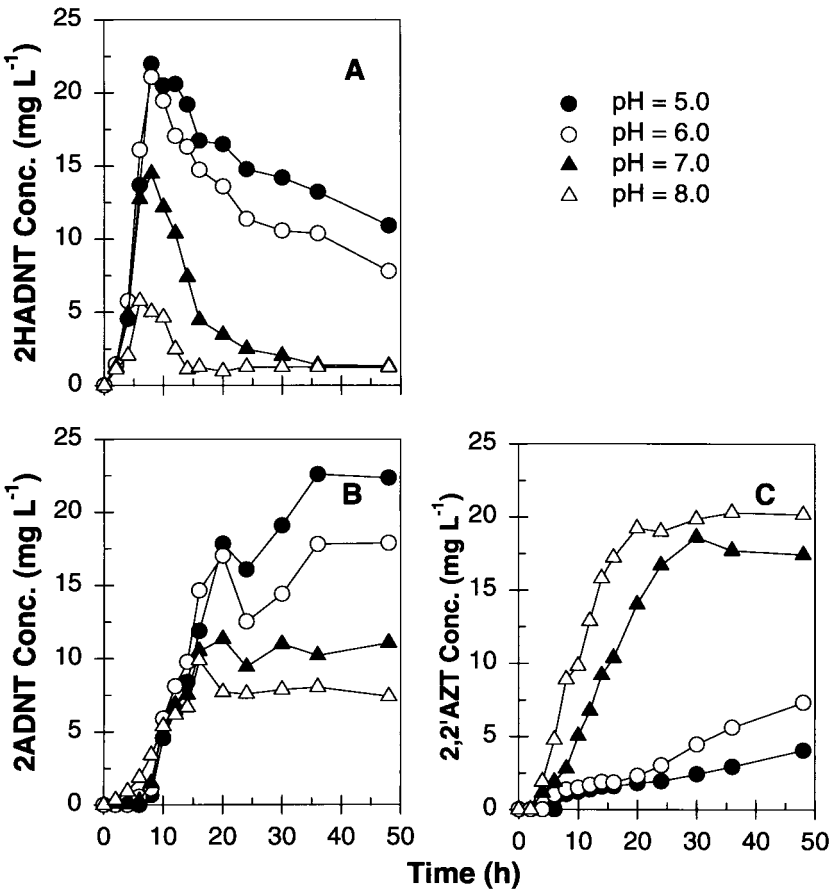

Figure 6. Influence of pH on the production of 2HADNT (A), 2ADNT (B), and 2,2′AZT (recovered from the cell fraction) (C).

8.0 than at pH 5.0 (data not shown). Nonsignificant mineralization of ^{14}C-TNT to $^{14}CO_2$ is consistent with prior research on TNT biodegradation by *Pseudomonas* spp. (Boopathy et al. 1994; Martin et al. 1997; Esteve-Núñez & Ramos 1998; Vasilyeva et al. 2000). The slightly increased turbidity of the culture medium containing TNT may have resulted from the formation of metabolites, such as ADNTs and AZTs. Nitrite concentration increased in the media during the incubation and was maximum when microbial growth reached the stationary phase. At this point, TNT in the medium was completely transformed. Small static background concentrations of nitrite were present in the control without strain MX, indicating that crystallized preparations of TNT contain nitrite. Previous research has shown nitrite is released from TNT (McCormick et al. 1976; Boopathy et al. 1994; Martin et al. 1997; Kalafut et al. 1998, Esteve-Núñez &

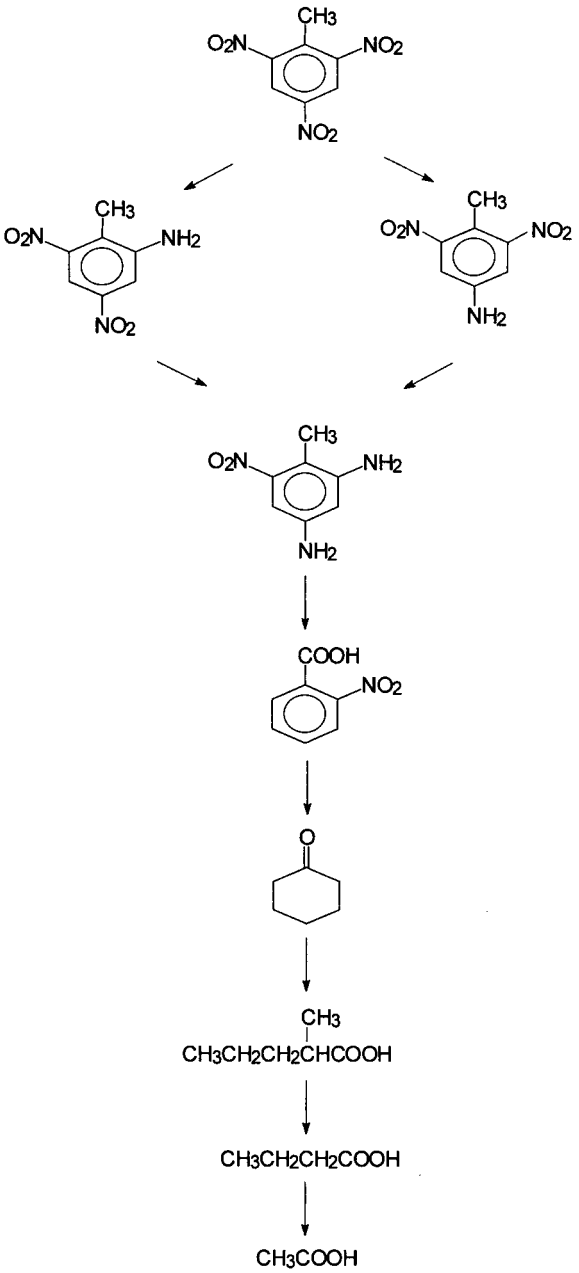

Figure 7. TNT degradation pathway proposed by Chung (Darling Marine Center, University of Maine, personal communication).

Ramos 1998; Esteve-Núñez et al. 2001); nitrophenols (Simpson & Evans 1953), and nitrobenzoic acids (Cartwright & Cain 1959; Esteve-Núñez et al. 2001), but the mechanism of denitration is uncertain.

The decrease in CFU after incubating *Pseudomonas aeruginosa* strain MX with TNT for 12 h may be attributed to toxicity from some of the accumulating TNT transformation products. The apparent decrease in toxicity of the substituted aromatics with increasing polarity may be due in part to strong binding to surface polysaccharides and reduced entry into the cell. Pure AZT did not significantly affect the growth of *P. aeruginosa* strain MX, which was consistent with comparatively low AZT toxicity (Collie et al. 1995), and may be attributed in part to low bioavailability (Vasilyeva et al. 2000). TNT metabolism to 3,5DNA, however, appears to increase toxicity in the culture medium, as previously reported for aquatic organisms (Talmage et al. 1999). In the soil environment, however, strong binding can significantly decrease the toxicity of hydroxylamino, amino, and AZT derivatives of TNT (Funk et al. 1993; Daun et al. 1998; Hughes et al. 1998; Achtnich et al. 1999). Wang et al. (2002) further suggested the incorporation of nitroaromatics into humic substances could be a possible tool for their removal.

Electron micrographs indicated that TNT and its metabolites affected the nuclear material of *P. aeruginosa* strain MX and inhibited cell division. This feature was similar to other reports of condensation, compacting, and margination of nuclear chromation in the presence of toxic compounds (Shneyvays et al. 1998; Shimojo et al. 1999). The decrease in cell counts of *P. aeruginosa* strain MX may have resulted from DNA component damage; however, the specific mechanism of toxicity has not been determined.

The rate of TNT transformation by *P. aeruginosa* strain MX decreased as it entered and remained in the stationary phase, which may be due to nutrient depletion and a reduction in the microbial population. A decrease in 2,2′AZT of about 25% in culture medium was not due to further transformation but to adhesion of 2,2′AZT to bacterial cells which was poorly extracted with buffer. Transformation of 2HADNT occurred abiotically as well as biotically; however, the main product of abiotic transformation was AZTs while biotic transformation produced mainly ADNTs. TNT reduction to HADNTs in the presence of *P. aeruginosa* strain MX appears enzymatic; however, AZT production from nitroso-DNTs and HADNTs may be due to an abiotic condensation reaction. The pH-dependency of AZT production is consistent with previous research (Funk et al. 1993; Khan et al. 1997). Strong binding of HADNTs (Nishino & Spain 1997; Daun et al. 1998; Achtnich et al. 1999), and the low availability of AZTs (Vasilyeva et al. 2000) would promote detoxification in soil.

Aside from reduction to ADNTs, metabolism of HADNTs to dihydroxylamino nitrotoluenes, DANTs, 3,5-dinitroaniline, aminonitrotoluene (ANT), cresols, or other products has been reported (Duque et al. 1993; Fiorella & Spain 1997; Esteve-Núñez & Ramos 1998; Hughes et al. 1998; Kalafut et al. 1998; Esteve-Núñez et al. 2001). In our experiments, detection of cyclohexanone further indicated the potential for TNT metabolism to less toxic and more biodegradable products. The transient appearance of 2,6DANT and 2,4DANT; dinitro- and nitrotoluene; and 3,5-dinitroaniline, along with nitrite release, indicated various routes of microbial metabolism and detoxification of TNT. Our observations suggest that successful bioremediation can be achieved by promoting the activity of microorganisms that are indigenous to the contaminated site.

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