Products of Anaerobic 2,4,6-Trinitrotoluene (TNT) Transformation by *Clostridium bifermentans*†

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Experiments to elucidate the 2,4,6-trinitrotoluene (TNT)-transforming activity of *Clostridium bifermentans* LJP-1 identified reductive TNT transformations that ultimately produced as end products triaminotoluene (TAT) and phenolic products of TAT hydrolysis. An adduct of TAT, apparently formed by condensation of TAT and pyruvic aldehyde (methyl glyoxal), was also detected.

Since the mid-1970s, biotransformation of nitroaromatic compounds has been studied to enable development of remediation technologies and predict the environmental fate of nitroaromatic contaminants (7). Both aerobic and anaerobic bacterial transformations have been studied to find ways to exploit the biochemical incorporation of molecular oxygen (25) or to avoid oxidative polymerizations (10). Studies of bacterial transformation of 2,4,6-trinitrotoluene (TNT) have shown reduction of the nitro groups to be a ubiquitous activity carried out by constitutively expressed cell constituents (16), probably representing fortuitous reactions between biological reductants and TNT, whose standard redox potential is relatively high (12). Other transformation reactions include an acetylation of the TNT reduction product, 2,4-diamino-6-nitrotoluene (DANT), to give 4-acetamide-2-amino-6-nitrotoluene (1, 11), an apparent dead-end product. These processes do not completely degrade TNT, and toxicity may persist while the aromatic nucleus and nitrogen substituents are present.

Reactions that remove the nitrogen substituents of TNT or its reduction products include an aerobic process presumably involving hydride-Meisenheimer complexes and the release of nitrite, ultimately giving toluene (20). Anaerobic TNT transformation studies indicating nitrogen removal have included product analyses suggesting reductive (2) and hydrolytic (10) mechanisms. Reductive mechanisms, effected by a sulfate-reducing bacterium, presumably reduce TNT to triaminotoluene (TAT), and TAT is subsequently reductively deaminated to give toluene. This speculative pathway identified neither TAT nor the intermediates between TAT and toluene. Hydrolytic mechanisms were proposed by Funk et al. (10), who observed methylphloroglucinol (MPG) and *p*-cresol in anaerobic incubations of a consortium from a munitions-fed soil-sewage sludge bioreactor. Their proposed pathway (6) requires further verification. Isolates of *Clostridium bifermentans* with rapid TNT transformation activities (21, 23) have been identified from this consortium. Comparisons of these isolates with other obligately anaerobic, fermentative bacterial cultures unexposed to TNT have shown that rapid TNT transformation is a general property of clostridia (8). Here we describe the TNT

transformation activities of one bioreactor isolate, strain LJP-1.

TNT transformation by *C. bifermentans* has been studied with actively growing cultures (21, 23) in which complex growth medium formulations including yeast extract or tissue infusions were used to promote maximal growth of this somewhat fastidious organism. When brain heart infusion (BHI) (Difco, Detroit, Mich.) was used in a study of cometabolic transformation of [ring-¹⁴C]TNT prepared by two-step nitration from toluene and [ring-UL-¹⁴C]toluene (specific activity, 58.9 μ Ci/mmol; radiochemical purity, >99% by high-performance liquid chromatography [HPLC]; Sigma Chemical Co., St. Louis, Mo.), TNT was completely transformed and the radioactivity was recovered in the liquid volume (101.7% $\pm$ 1.3% of the total added radiolabel [mean $\pm$ standard deviation]; n = 3). Nearly all the recovered radioactivity was soluble at the end of vegetative growth (99.2% $\pm$ 1.1%) (filtrate from 0.2- μ m-pore-size filter; 25-mm-diameter syringe filters) (Supor; Gelman Sciences, Ann Arbor, Mich.), indicating that TNT was not mineralized by LJP-1 cometabolically and that only small quantities of the aromatic carbons could have been assimilated.

Defined media can support growth of *C. bifermentans* but must contain several amino acid and vitamin supplements (14). To avoid interference by organic components of the medium, M9 defined medium (18) without ammonium or added reducing agent was used with suspensions of actively growing cells of LJP-1 (A_{600} , 0.7 to 0.9; cells obtained from BHI agar; atmosphere of 90% N₂, 10% H₂). TNT concentration and metabolite production were monitored by HPLC (4.6-mm by 25-cm C₁₈ column; Microsorb; Rainin Instrument Co., Emeryville, Calif.); acetonitrile (A) and 15 mM potassium phosphate, pH 7.1 (B), as eluting solvents; flow rate, 1 ml/min; 100- μ l injection volume; 95% B (0 to 7 min) to 50% B (7 to 12 min, held for 2 min) to 30% B (14 to 15 min, held for 5 min), followed by a return to initial conditions (22 min); detection at 210 nm with peak-actuated scanning from 200 to 600 nm. Samples were removed anaerobically to avoid exposure to oxygen and filtered before analysis. Standards of DANT, 2-amino-4,6-dinitrotoluene (2ADNT), and 4-amino-2,6-dinitrotoluene (4ADNT) were purchased from SRI International (Menlo Park, Calif.); 2,6-diamino-4-nitrotoluene was purchased from Aldrich (Milwaukee, Wis.); and TAT trihydrochloride was purchased from ChemService, Inc. (West Chester, Pa.). TNT was prepared from 2,4-dinitrotoluene (Eastman Organic Chemicals, Rochester, N.Y.) by nitration (procedure

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FIG. 1. Time course of [^{14}C]TNT transformation by a cell suspension of LJP-1. Samples from a cell suspension of LJP-1 which received 50 mg of [^{14}C]TNT per liter were filtered and analyzed by HPLC. Fractions were collected, and disintegrations per minute corresponding to various product fractions were added together and expressed as a percentage of the total amount of added radiolabel. HPLC fractions were TNT (20 to 22 min) (◆); DANT, AHANT, and DHANT (16 to 18 min) (◇); TAT (6.5 to 8.5 min) (■); phenolic products (8.5 to 10.5 min) (▲); and a single peak with A_{317} (15 to 16 min) (●). An example of one such chromatogram is shown in Fig. 2.

modified from that described in reference 13). 4-Hydroxylamino-2,6-dinitrotoluene (HADNT), 2,4-dihydroxylamino-6-nitrotoluene (DHANT), and 2-hydroxylamino-4-amino-6-nitrotoluene (AHANT) were obtained from Paul Fiorella, Tyndall Air Force Base, Fla., who identified these compounds by mass spectroscopy (MS) and nuclear magnetic resonance (NMR) (9). TNT, ADNT, and DANT were stable in the suspension medium in the absence of cells. In cell suspensions without an added energy source, ≈ 2 h was required to remove 80 ppm of TNT (one experiment), with aminodinitrotoluenes (predominantly 4ADNT) and HADNT appearing as final products. Significant cell lysis occurred after ≈ 4 h of incubation at room temperature. Using [ring- ^{14}C]TNT, we recovered $99.6\% \pm 1.8\%$ of the total radioactivity in the liquid volume; $80.8\% \pm 2.2\%$ was soluble after 24 h ($n = 3$).

When glucose-supplemented (0.2%, wt/vol) suspensions of LJP-1 were used, reduction of TNT nitro groups was shown by the transient appearance of HADNT (data not shown), 4ADNT, DHANT, AHANT, and DANT and the accumulation of TAT (Fig. 1). Products eluted earlier than TAT, represented by at least three chromatographic peaks, appeared transiently before TAT production was maximal. These collectively accounted for $50.3\% \pm 6.2\%$ of the total added radioactivity at 1 and 2 h of incubation (two experiments, four datum points). After 8 h of incubation, TAT represented the major product of TNT transformation and made up $44.0\% \pm 3.8\%$ of the total added radioactivity after 22 h ($n = 3$). TNT transformation was much faster in the glucose suspension than in suspensions without an added energy source; no TNT was

detected after 20 min of incubation with TNT at an initial concentration of ≈ 50 ppm (Fig. 1). Much of the total mass of TNT initially added to glucose-supplemented cell suspensions was not accounted for in the initial sample, however. In one experiment, a filtered sample taken less than 1 min after TNT addition contained 52% of the total amount of added radioactivity; in another, only 19% was recovered after filtration. In later samples, $98.1\% \pm 2.3\%$ of the radioactivity was recovered after filtration and $92.0\% \pm 1.7\%$ of the applied radioactivity was recovered in HPLC effluent ($n = 5$).

At the end of experiments such as that shown in Fig. 1, $80.9\% \pm 11.1\%$ of the total added radioactivity could be accounted for in three chromatographic fractions ($n = 3$) (an example is shown in Fig. 2). The largest percentage was in the fraction identified as TAT. The product fraction eluted next contained at least two chromatographic peaks, with the first (the earliest eluting) predominating (Fig. 2). These peaks were also seen in solutions of TAT trihydrochloride which had decomposed because of lack of pH buffering. These products gave molecular ions of m/z 138 by particle beam HPLC-MS performed on a Hewlett-Packard model 1050 HPLC with a 2.0-by 25-cm C_{18} column (Spherex) and a 2.0-by 5-cm guard column (Phenomenex, Torrance, Calif.); acetonitrile (A) and 15 mM ammonium acetate, pH 7.1 (B); flow rate, 0.3 ml/min; 99% B (0 to 5 min) to 90% B (5 to 7 min) to 55% B (7 to 27 min, held for 3 min), return to starting conditions (32 min); detection by A_{220} as well as scanning ion monitoring with a model 5989A quadrupole MS equipped with a model 59980B particle beam interface. Gas chromatography (GC)-MS anal-

FIG. 2. Elution profile of [^{14}C]TNT transformation products. Samples were analyzed as described in the legend for Fig. 1, and the chromatogram (solid line) and disintegrations per minute in eluant fractions (bars) are shown. The sample used was from the experiment represented in Fig. 1 after 22 h of incubation.

ysis of the trimethylsilyl (TMS) derivatives of decomposed TAT and TNT-transformation products gave corresponding retention times and molecular ions of m/z 354, as predicted for fully derivatized diaminocresol isomers. GC-MS was performed with a 5890 series II GC equipped with a model 7673 autoinjector and a 0.2-mm (inside diameter) by 25-m DB-5ms column (J&W Scientific, Folsom, Calif.). Helium was used as the carrier gas at 100-kPa column head pressure; 1- μl splitless injection; inlet, 280°C; column oven, 100°C for 2 min to 320°C at 10°C/min and held for 2 min; MS interface, 300°C. TAT and its decomposition products were derivatized by dissolving solid samples in anaerobic pyridine and adding 0.1 ml of the respective solution to 0.4 ml of bis-(trimethylsilyl)acetamide (BSA), or bis-(trimethylsilyl)trifluoroacetamide plus 1% trimethylchlorosilane (BSTFA + 1% TMCS) (Pierce Chemical Co., Rockford, Ill.). Derivatization reaction mixtures were incubated at 60°C for 20 min before injection into the GC. Aqueous solutions were dried by freeze-drying. These products, and those observed in aqueous, unbuffered TAT trihydrochloride solutions, showed a shift of UV absorbance maxima to shorter wavelengths (from 220 nm for TAT to 215 nm). ^1H -NMR of the TAT decomposition products showed aromatic resonances (7.1, 7.3, and 7.5 ppm, singlets) downfield of the TAT aromatic resonance (6.7 ppm, singlet; $[\text{D}_6]$ dimethyl sulfoxide [99.9 atom% D] with 0.03% [vol/vol] TMS [Aldrich]; IBM NR300 spectrometer at 300 MHz). When TAT was dissolved in H_2^{18}O (95 to 98% ^{18}O ; Cambridge Isotope Laboratories, Andover, Mass.), incorporation of one ^{18}O atom was seen for each of the respective HPLC and GC peaks. These data are consistent with the replacement of an amino group of TAT by a hydroxyl group. Solutions of ≈ 1 mM TAT trihydrochloride dissolved in deionized water ranged in pH from 3.5 to 4.1, a range previously shown to effect the decomposition of TAT (19) in studies indicating that TAT decomposed with release of approximately 1 mol of ammonium per mol of TAT in acidic solutions, a finding also consistent with hydrolysis.

The dependence of TAT trihydrochloride stability in aqueous solutions upon adequate pH buffering and the use of an unverified synthesis procedure for 2,4-diamino-6-hydroxylaminotoluene (DAHAT) (19) previously led us to incorrectly identify the TAT chromatographic peak as DAHAT (17). The

protocol initially used to synthesize DAHAT included zinc powder and a small amount of ammonium chloride to reduce DANT (19). The removal of starting material, requiring ≈ 2.5 h with continuous stirring, was monitored by HPLC. When we compared this procedure with palladium- H_2 reduction of 2,6-diamino-4-nitrotoluene, which had been used to synthesize TAT (19), in our hands both the Zn^0 -DANT and the Pd-H_2 -2,6-DANT reactions gave a single detectable product with the same HPLC retention time and UV-visible absorption spectrum as those obtained from TAT trihydrochloride prepared in M9 buffer. These products gave a molecular weight of 137 by HPLC-MS or GC-MS of TMS derivatives, which corresponds to TAT and not to DAHAT. Reduced methyl viologen was also reported to reduce DANT to give DAHAT (19). This procedure gave a different product which was well resolved from TAT by our HPLC method, but we were unable to obtain mass spectra for this material.

The occurrence of phenolic products in common with products of TAT hydrolysis reactions suggested that hydrolysis had taken place in the cell suspensions, but incubations of TAT with cell suspensions or spent filtrate did not produce significant amounts of these phenolics (final pH ≥ 6.4). Incubations of cell suspensions with 4ADNT instead of TNT yielded more of the first phenolic HPLC peak than did incubations with TNT. Incubations with 2ADNT gave smaller amounts of phenolic products than did incubations with TNT, and the second peak predominated. Incubations with DANT yielded very small amounts of phenolic products.

In addition to TAT and phenolic products, a third fraction containing one chromatographic peak with absorbance maxima at 237 and 317 nm accounted for a significant proportion of the final products. This material was also seen in incubations with DANT or TAT in place of TNT (8, 17). As an abiotic control, the peak was not observed when TAT was incubated in spent suspension medium from which the cells had been removed by filtration, nor was the material observed in incubations of cell suspensions with phenolic hydrolysis products of TAT, indicating that it was formed from TAT and not its decomposition products. ^{14}C radiolabeling confirmed that this material was derived from TNT and accounted for $12.8\% \pm 4.8\%$ of the total TNT transformation ($n = 3$). MS analysis

gave a molecular weight of 191 (Fig. 3). The relative abundance of the ion at $M+1$ (measured by electron impact ionization) was $12.13\% \pm 0.39\%$, a quantity consistent with seven molecular formulas containing carbon, hydrogen, nitrogen, and/or oxygen (24). Experiments using methyl- D_3 , deuterium-labeled TNT (synthesized by two-step nitration of [methyl- D_3]toluene, 98 atom% D; Cambridge), indicated that all benzylic protons of TNT were present in this product and that the major fragment ion, which corresponded to the loss of a methyl group, did not result from loss of the benzylic methyl (Fig. 3). When [$^{15}N_3$]TNT (synthesized in a two-step procedure from toluene and $NH_4^{15}NO_3$; ≥ 98 atom% ^{15}N ; Cambridge) was used in the cell suspension experiments, the unidentified peak gave a molecular ion at m/z 194 (Fig. 3), indicating that all original nitrogen atoms were retained and eliminating molecular formulas with fewer than three nitrogen atoms. Three formulas were left as potential solutions: $C_{10}H_{13}N_3O$, $C_9H_{11}N_4O$, and $C_{10}H_{15}N_4$. These data indicated that TAT had been chemically modified by the cell suspensions to give a net increase of 54 atomic mass units.

Only a few structural formulas based on TAT seemed theoretically feasible for explaining the data indicating additional mass. One of these was a Schiff base with a C_3 ketone group bonded to a nitrogen atom of TAT (Fig. 4). It seemed possible to synthesize this compound by allowing condensation of pyruvic aldehyde with TAT to form the Schiff base. Simple incubations of these two components in M9 buffer solution yielded the same chromatographic peak, with equimolar pyruvic aldehyde (Aldrich) and TAT concentrations giving the maximal yield. In addition to HPLC retention time, the UV-visible and mass spectra of the material synthesized from TAT and pyruvic aldehyde were indistinguishable from those of the unknown TNT transformation product. Other peaks eluting later were seen with higher relative concentrations of pyruvic aldehyde and had mass spectra corresponding to addition of another 54 atomic mass units to the single Schiff base to give a double Schiff base. Certain of those HPLC peaks were also seen in some longer incubations of LJP-1 with TNT or DANT.

In an experiment to determine if the TAT Schiff base was a dead-end product or one that might be transformed further by growing cultures, a cell suspension was used to produce the material from [^{14}C -ring]TNT. Dehydrated BHI was then added, and the suspension was filter sterilized. One aliquot was kept sterile, another was inoculated with LJP-1, and both were incubated overnight at 37°C. HPLC analyses comparing the two treatments indicated that no significant net removal of the 317-nm-absorbing peak was seen. A new, more polar, radiolabeled product fraction was seen only in the inoculation experiment and in growing cultures with [^{14}C]TNT.

The capacity of *C. bifermentans* for rapid TNT nitro group reduction is apparent from this study and previous ones (8, 17). The physiology of this organism, an obligate fermentative anaerobe that uses phosphoroclastic reactions for ATP generation, leads to the accumulation of low-potential reductants through glucose fermentation. These include reduced forms of pyruvate:ferredoxin oxidoreductase, ferredoxin, and hydrogenase. Two of these iron-sulfur protein redox mediators (ferredoxin and hydrogenase) are known to reduce TNT and its more reduced derivatives such as DANT (19). In this respect, the cells serve as effective catalysts for the transfer of reducing

equivalents from glucose to nitro, nitroso, and hydroxylamino groups. The extent of nitro group reduction is probably related to the midpoint potential of suitable redox centers, and the rate of reduction is probably related to their concentration and the metabolic turnover of reducing equivalents. The transient appearance of hydroxylamino and dihydroxylamino intermediates from TNT suggests that reduction of nitro groups to form amino groups is somehow rate limited by the final two-electron step. Since the reduction activity catalyzed by *C. bifermentans* requires fermentable carbon sources, the role *C. bifermentans* may play in our reactor fed only munitions compounds is uncertain.

Transformations other than reduction have also been seen in *C. bifermentans*. Phenolic products that also result from the hydrolysis of TAT were identified in cell suspensions which had transformed TNT or aminonitrotoluenes, but they were not produced in significant amounts by cells exposed to TAT or DANT. The exact source of the phenolics cannot be determined from the present data, but experiments with 4ADNT have shown it to be a better substrate for their production than TNT. The mechanism of phenolic production during TNT transformation is of interest since removal of nitrogen-containing groups may allow metabolism through known catabolic pathways (4, 15). In this respect, the transformation carried out by *C. bifermentans* is incomplete, since MPG, the product of complete hydrolytic deamination of TAT, was not seen. Therefore, if MPG is a significant product or intermediate of TNT transformation by the anaerobic consortium (10), then other organisms, or activities not displayed in the present study, are required.

In addition to phenolic products, another product results from a novel chemical modification that occurs during TNT transformation with *C. bifermentans* and several other clostridia (8). The properties of this product are consistent with a Schiff base formed from TAT. Although the material could be synthesized from TAT and pyruvic aldehyde, it is not known how it may be formed by clostridia. The presence of aromatic amines may stimulate the modification as a detoxification mechanism, or the xenobiotics may cause some unusual side reactions of the fermentative pathway. Acetylation of DANT by *Pseudomonas fluorescens* has been ascribed to reaction between activated acetate (acetyl coenzyme A) and DANT (11). Again, however, it is not known if this response is specifically for detoxification or is simply fortuitous. The greater production of the Schiff base in cell suspensions than in growing cultures (data not shown) and the absence of any avid activity by the growing cultures leading to its removal indicate that the modification is somehow promoted by conditions of nutrient limitation. Pyruvic aldehyde (methyl glyoxal) has been observed in nitrogen-limited cultures, and in cultures exposed to other physiological stresses, during glucose fermentation (5). The observation that other, more polar products appeared in growing cultures indicates that still other modifications are possible when ample nutrients are available.

Our studies with cell suspensions and growing cultures of clostridia allow us to speculate on the fate of TNT in environments where clostridial activities prevail and on the role of clostridia in our munition-fed bioreactor. Since clostridia are common in soils, the addition of fermentable sugars to soils contaminated with TNT would be expected to stimulate the

FIG. 3. Mass spectra of the 317-nm-absorbing TNT transformation product. (A) MS of material obtained from the LJP-1 cell suspension with TNT using particle beam HPLC-MS and electron impact ionization; (B) same as panel A except with [methyl- D_3]TNT; (C) same as panel A except with [$^{15}N_3$]TNT; (D) same as panel A except using chemical ionization with methane as the ionization gas.

FIG. 4. Proposed structural formula for the 317-nm-absorbing TNT transformation product.

types of reactions observed in our experiments, which would lead primarily to the formation of TAT with some phenolic and Schiff base derivatives. The likely fate of TAT has been proposed by Rieger and Knackmuss (22) to include polymerization, due to autooxidation, and sorption to soil particles. The secondary products described here may not differ greatly from TAT in these respects, since we noticed a rapid darkening of purified material upon exposure to air, indicating the susceptibility of these products to oxidative polymerization (unpublished observation). The sorptive character imparted by amine substitution also remains in these species. In consortia, however, TAT, the more oxidized species that precede it, and the material derived from it might be amenable to aerobic mineralization, a possibility supported by studies showing TNT mineralization in soils in which nitro group reduction products were the only intermediates identified (3). Thus, in obligately anaerobic mixed cultures fed fermentable substrates (10), clostridia probably play an important role in the complete or near-complete reduction of TNT. Other microbial groups, however, appear to influence the ultimate transformation of reduced TNT metabolites to their final products.

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