Appl Microbiol Biotechnol (2005) 67: 397–404
DOI 10.1007/s00253-004-1736-x

Hong Yin · Thomas K. Wood · Barth F. Smets

Reductive transformation of TNT by *Escherichia coli*: pathway description

Received: 9 June 2004 / Revised: 10 August 2004 / Accepted: 31 August 2004 / Published online: 13 October 2004

Abstract The reductive transformation of 2,4,6-trinitrotoluene (TNT) was studied using aerobically grown *Escherichia coli* cultures. In the absence of an external carbon or energy source, *E. coli* resting cells transformed TNT to hydroxylaminodinitrotoluenes (2HADNT, 4HADNT, with 4HADNT as the dominant isomer), aminodinitrotoluenes (4ADNT, with sporadic detection of 2ADNT), 2,4-di(hydroxylamino)-6-nitrotoluene (24D(HA)6NT), 2,4-diamino-6-nitrotoluene (24DA6NT), and an additional compound which was tentatively identified as a (hydroxylamino) aminonitrotoluene isomer via gas chromatography/mass spectroscopy and spectral analysis. The resting cell assay, performed in an oxygen-free atmosphere, avoided formation of azoxy dimers and provided good mass balances. Significant preference for reduction in the *para* versus *ortho* position was detected. The formation of 24D(HA)6NT, but not ADNT, appeared inhibited by the presence of TNT. The rate and extent of TNT reduction were significantly enhanced at higher cell densities, or by supplying an exogenous reducing power source, revealing the importance of enzyme concentration and reducing power. Whether the oxygen-insensitive *E. coli* nitroreductases, encoded by *nfsA* and *nfsB*, directly catalyze the TNT reduction or account for the complete TNT transformation pathway, remains to be determined.

H. Yin · B. F. Smets
Department of Civil and Environmental Engineering & Molecular and Cell Biology, University of Connecticut, Storrs, CT 06269, USA

T. K. Wood
Department of Chemical Engineering & Molecular and Cell Biology, University of Connecticut, Storrs, CT 06269, USA

B. F. Smets (✉)
Department of Environment and Resources, Building 113, Technical University of Denmark, DK-2800 Kgs. Lyngby, Denmark
e-mail: bfs@er.dtu.dk
Tel.: (+45) 45 25 16 00
Fax: (+45) 45 93 28 50

Introduction

2,4,6-Trinitrotoluene (TNT) is used extensively in the production of explosives, dyestuffs, and photographic chemicals (Sax and Lewis 1987). It can cause severe contamination of soil and water and is mutagenic and potentially carcinogenic (Honeycutt et al. 1996). Thus, the remediation of TNT-contaminated sites is of great interest for the protection of human and ecosystem health.

The highly polarized electronegative N–O bond makes a nitro group in TNT easily reduced, yet TNT is poorly mineralized, probably due to combined steric and electrophilic effects of multiple nitro substitutions (Esteve-Nunez et al. 2001). Even aerobic bacteria tend to transform TNT by reduction of nitro groups, but reduction beyond diaminonitrotoluenes is typically not observed (Fuller and Manning 1997). The presence of oxygen furthermore promotes coupling reactions of partial TNT reduction products (e.g., formation of azoxytetranitrotoluenes), preventing mineralization (Haidour and Ramos 1996). A few aerobic strains display an alternate reductive TNT pathway that involves a Meisenheimer complex (Esteve-Nunez et al. 2001; French et al. 1998).

Under anaerobic conditions, reduction of TNT is faster and more complete than under aerobic conditions. The TNT reductive pathway involves sequential transformation of the nitro groups to the corresponding amines via hydroxylamino intermediates (Esteve-Nunez et al. 2001). It has been observed that reduction of the second nitro group does not begin until the first nitro group has been converted to an amine; and reduction of the third nitro group requires very low oxidation reduction potential (≤ -200 mV) (Spain et al. 2000; Esteve-Nunez et al. 2001). As electron-deficient nitrogen atoms are reduced, the molecule becomes less susceptible to reduction and reaction rates decrease. To date, there is no conclusive evidence of a TNT mineralization pathway over triaminotoluene (TAT).

Reductive transformation of TNT has been documented for many bacterial genera, e.g., *Desulfovibrio* spp (Boopathy and Manning 1996), *Myriophyllum aquaticum* (Wang et al. 2003), *Clostridium* spp (Ederer et al. 1997), *Lactobacillus*

spp (Ederer et al. 1997), *Pseudomonas* spp (Fiorella and Spain 1997), *Actinomycete* sp. (Pasti-Grigsby et al. 1996), and *Salmonella typhimurium* (Ederer et al. 1997), and for many pure enzymes (Fiorella and Spain 1997; Watrous et al. 2003). Surprisingly, no comprehensive study on the reductive transformation of TNT by *Escherichia coli* has been reported, although it has long been documented that *E. coli* cell extracts can reduce TNT to hydroxylaminodinitrotoluenes [4-hydroxylamino-2,6-dinitrotoluene (4HA26DNT), 4-amino-2,6-dinitrotoluene (4A26DNT)] and 4,4′-azoxytoluene (4,4′-AZT), while live cultures of *E. coli* can yield 2, 4-diamino-6-nitrotoluene (24DA6NT) (Ederer et al. 1997; McCormick et al. 1976). It is informative to know the reductive pathway, since recombinant *E. coli* strains might be developed and used to remediate nitroaromatic compounds.

The goal of this study was to comprehensively evaluate the reductive TNT transformation pathway in *E. coli*. Of specific interest was the detection of pathway intermediates and elucidation of the key biological variables that govern the rate and extent of reduction. This work represents the first step in developing a comprehensive whole-cell-level understanding of nitroarene transformation in *E. coli*. Specifically, we were able to confirm 4A26DNT and 24DA6NT, discovered 2,4-di(hydroxylamino)-6-nitrotoluene [24D(HA)6NT] and a compound tentatively identified as a (hydroxylamino)aminonitrotoluene isomer (2HA4A6NT) as pathway intermediates, quantified a preference (above 10:1) for reduction of the *para* versus the *ortho* position, and observed that the rate and extent of TNT reduction could be controlled by the concentration of biocatalyst and exogenous reducing power.

Materials and methods

Chemicals

TNT was purchased from Chemservice (West Chester, Pa.). Analytical standards of TNT, 2-hydroxylamino-4,6-dinitrotoluene (2HA46DNT), 2-amino-4,6-dinitrotoluene (2A46DNT), 4HA26DNT, 4A26DNT, 24DA6NT and 2,2′, 6,6′-tetranitro-4,4′-azoxytoluene were obtained from AccuStandard (New Haven, Ct.). 24D(HA)6NT was a generous gift from Dr. Hughes (Georgia Tech, Atlanta, Ga.).

Microorganism, media, and culture conditions

For inocula, *E. coli* JM 109 (ATCC 53323)/pGEM4Z (Promega) was cultured in Luria–Bertani broth overnight at 37°C and aerated via agitation at 150 rpm. Cells were regrown under the same condition at an inoculum:medium ratio of 1:15 (v/v).

Transformation of TNT

Anaerobic TNT transformation assays were performed in a chamber filled with anaerobic gas (N_2 85%, H_2 5%, CO_2 10%) or pure N_2 gas. *E. coli* cells were harvested in the exponential growth phase by centrifugation at 8,110 *g* and 4°C for 10 min when the culture optical density at 600 nm (OD_{600}) was 1.0. The cell pellets were washed twice with sodium phosphate buffer (PBS; 100 mM, pH 6.5) and resuspended in PBS by adjusting the OD_{600} to the required value. The TNT transformation assay was initiated by adding a small amount of TNT stock solution (1 mM in acetonitrile) to the culture at 30°C and immediately mixing by vortexing. Control experiments were performed by adding TNT to acid-killed cell suspensions (1 N HCl). At a defined time after adding TNT, 1-ml aliquots were removed, mixed with 50 μl of 1 N HCl (to stop microbial activity) and 5 μl of 100 mM ascorbate (to minimize auto-oxidation). Cell pellets were removed by spinning at 11,122 *g* for 5 min and the supernatant was removed for high-performance liquid chromatography (HPLC) analysis. At least 10-ml samples were taken for extraction of metabolites before gas chromatography/mass spectroscopy (GC/MS) identification. Metabolites formed were confirmed by HPLC retention time, ultraviolet/visible (UV/Vis) spectra, and/or fragmentation patterns of chemical standards.

Isolation/derivatization of metabolites

The samples for GC/MS analyses were obtained from cell suspensions incubated with TNT as described above. Cells were removed by centrifugation at 18,248 *g* and 4°C for 20 min. Culture supernatants were adjusted to pH 6.5 and extracted with equal volumes of ethyl acetate. The resulting aqueous phase was acidified to pH 2.5 with 1 N HCl and extracted again with equal volumes of ethyl acetate. The extracts were dried over anhydrous sodium sulfate and the excess solvent was evaporated under a continuous flow of nitrogen gas (Johnson et al. 2001; Spanggord et al. 1991). The extracted metabolites were dissolved in a small volume of acetonitrile before derivatization. Trimethylsilyl derivatives were prepared with *N*,*O*-bis(trimethylsilyl)-trifluoroacetamide (BSTFA) according to methods provided by the distributor of BSTFA (Aldrich, Mo.). Catechol was spiked to verify the quality of derivatization reagent and efficiency of derivatization on a daily basis.

Analytical methods

Reverse-phase HPLC analysis was performed with a Jasco chromatography workstation equipped with a photodiode array detector (PU980, MD1510; Jasco, Easton, Md.), a Spherisorb C8 column (25 cm long, 4.6 mm diam., 5 μm film; Alltech, Deerfield, Ill.), and isocratic elution (50% acetonitrile/50% deionized water) at 1 ml/min. 2,2′,6,6′-Tetranitro-4,4′-azoxytoluene was identified at 75% acetonitrile and a flow rate of 1 ml/min for 10 min. Absorbance spectra at 200–400 nm were collected to permit compound identification, while quantitation was performed at 254 nm. Isomers of hydroxylaminodinitrotoluene (HADNT) and aminodinitrotoluene (ADNT) were not well separated on

this column. Since the *para*-reduced isomer was the dominant isomer formed (see Results), the total amounts of HADNTs and ADNTs were quantified using standard curves of 4HA26DNT and 4A26DNT, respectively.

GS/MS analyses were done on a Saturn 3 MS and a Varian series 3400 GC with a HP-5 M.S. capillary column (30 m, long 0.25 mm diam., 0.25 μm film; Hewlett-Packard). Helium was the carrier gas at a constant flow rate of 1.0 ml/min; and the injector and transfer line temperature were 280°C. The chromatography program was an initial column temperature of 50°C for 1 min which was increased at 10°C/min to 280°C followed by isothermal operation for 1 min.

Nitrite concentrations were measured photometrically by the method of Griess–Ilosvay as modified by Shinn (Montgomery 1961). Protein concentrations were determined using the bicinchoninic acid protein assay, using BSA as standard (Pierce, Rockford, Ill.; Johnson et al. 2001)

Results

Enzymatic transformation

In control experiments with acid-killed *E. coli* cells, no TNT transformation was observed over 24 h (data not shown). Some evidence of TNT loss was observed after 2 days of incubation at 500 μM. No other transformation products were detected via HPLC analysis. Equilibrium concentrations were only obtained after approximately 110 min, potentially due to temporary sorption/precipitation of the added TNT (see Discussion). For all subsequent analyses with live cells, concentration profiles were normalized to paired abiotic control profiles.

Product identification during TNT reduction at different cell densities (single time-point, single TNT concentration)

To evaluate the importance of cell concentration on TNT reduction, TNT (100 μM) was contacted with incremental cell densities (OD_{600}=1.0, 2.5, 5.0, 7.2; protein concentration = 300, 750, 1,500, 2,160 mg/l) for 2 h. Reaction mixtures were extracted with ethyl acetate, BSTFA-derivatized, and the intermediates were identified by GC/MS. No nitrite or azoxytoluenes was detected throughout the reaction. Cell density had a great effect on the detected TNT transformation products. At higher cell density, more reduced TNT products were obtained. Only at the lowest cell density was residual TNT detected. At OD_{600}=2.5, 4HA26DNT, 2HA46DNT, and 4A26DNT were detected. Increasing the cell density to OD_{600}=5.0, two new compounds (I, II) were detected in addition to HADNTs and ADNT. At the highest density (OD_{600}=7.2), only 24DA6NT and compound II were detected in the supernatant.

Identification of compounds I and II

Compound I was confirmed as 24D(HA)6NT by comparing the retention time, UV spectrum, and mass fragmentation pattern with authentic 24D(HA)6NT (obtained from Dr. Hughes, Georgia Institute of Technology, Ga.; Fig. 1). Compound II has a HPLC retention time of 4.4 min. It elutes faster than 24DA6NT (5.3 min), but slower than 24D(HA)6NT (3.9 min). Its derivatized mass was 327 [327=183+(73×2)−2], yielding an underivatized mass of 183 [183=327−(2×73)+2], consistent with A(HA)NT (Fig. 1), which could be formed from reduction of either 24D(HA)6NT or one of the ADNT isomers. Given the trace and sporadic detection of the 2HA46DNT and 2A46DNT, this compound is most likely 2HA4A6NT.

Sequential product formation in TNT reduction

Clear evidence of sequential TNT reduction was observed when a single experiment (OD_{600}=2.5; C_i=100 μM) was periodically sampled up to 24 h. Both isomers of HADNT occurred initially, followed by both ADNT isomers (80 min). Subsequently, both compounds I and II [24D(HA)6NT, A(HA)NT] were detected (140 min), prior to detection of 24DA6NT (240 min).

Ratio of (4HA26DNT + 4A26DNT)/(2HA46DNT + 2A46DNT)

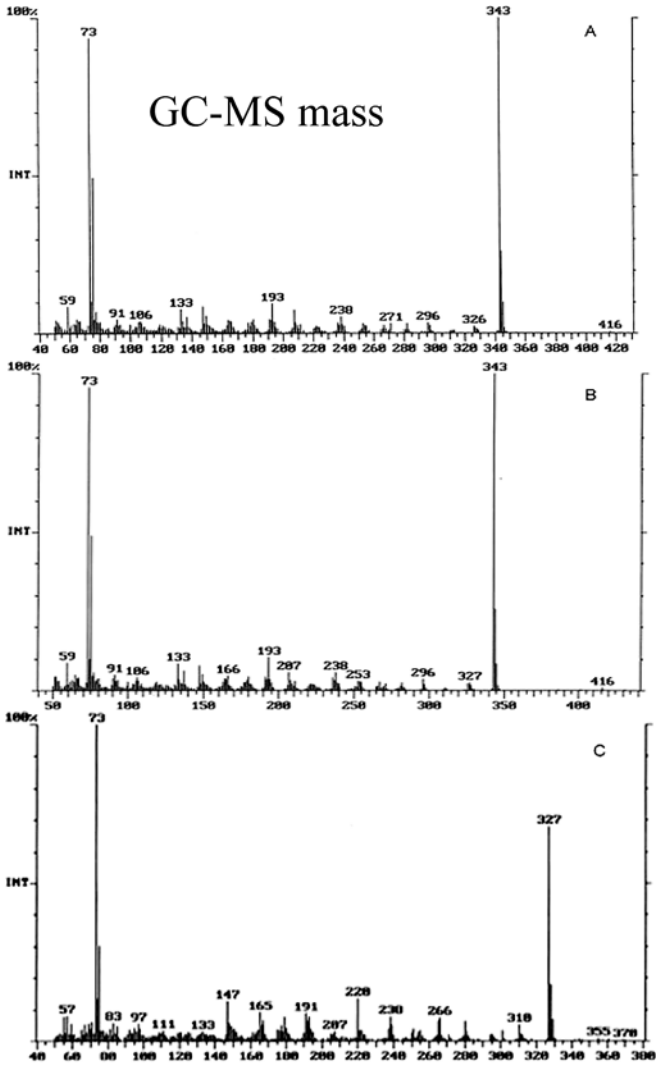
The ratio of the transformation product reduced in the *para* position (4HA26DNT + 4A26DNT) versus the one reduced in the *ortho* position (2HA46DNT + 2A46DNT) was consistently higher than 10:1 (data not shown), indicating the preferred reduction of the *para* position over the *ortho* position.

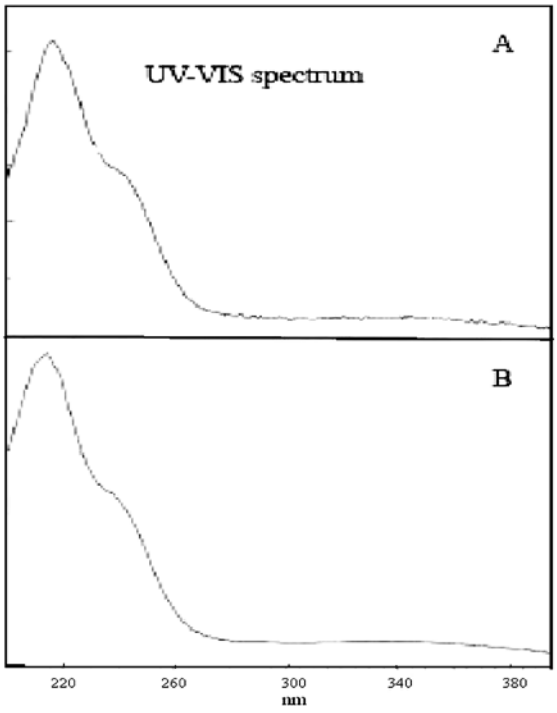
Effect of exogenous carbon source

In several preliminary experiments, we observed incomplete TNT reduction (especially under conditions of low cell densities, high TNT concentrations; data not shown). To examine whether such lack of activity could be enhanced, glycerol (20 mM) was added as an exogenous energy/electron source during the assay. In the presence of glycerol, much faster and more complete TNT transformation was observed, suggesting that internal reducing power or energy may be a limiting factor in TNT transformation (Fig. 2).

Quantitative analysis

Detailed time-course analyses (Fig. 3) revealed the effect of initial TNT concentration (C_i) on the extent of TNT





◀ **Fig. 1** UV-Vis spectrum of hydroxylamino compounds and GC/MS electron ionization mass spectra of trimethylsilyl derivative of hydroxylamino compounds. Compound I (**a**), authentic 24D(HA) 6NT (**b**), and compound II (**c**) were analyzed as described in the Materials and methods

reduction (all experiments were performed with identical cell density, without exogenous energy source; OD_{600}=1.0, cell protein = 300 mg/l). TNT concentrations decreased in all experiments (Fig. 3a). Individual TNT depletion curves revealed time-variant transformation rates with rates at initial time-points higher than at later time-points.

HADNT accumulation was observed in all treatments (Fig. 3b). The rate and extent of HADNT accumulation appeared inversely related to the initial TNT concentration. At C_i=40 μM and 70 μM, 89±4% of the transformed molar TNT concentration was transiently detected as HADNT (after 150 min, 1,140 min, respectively) against only 23% and 14% at C_i=270 μM and 500 μM (after 1,440 min). HADNT concentrations decreased after complete removal of TNT (Compare curves for 40 μM, 70 μM in Fig. 3a, b), indicating their continued transformation.

ADNT was slightly delayed from HADNT accumulation (no ADNT was detected at 50 min in any experiment; Fig. 3c). However, subsequent ADNT accumulation appeared concomitant with HADNT accumulation. In contrast to the HADNT profiles, higher initial TNT concentration did not impair ADNT accumulation rates. Instead, ADNT accumulation rates increased with increasing C_i. ADNT accounted for 21–25% of the transformed TNT after 1,500 min at C_i=70, 270, and 500 μM. For C_i=40 μM, only 10% of the transformed TNT was accounted for by the formed ADNT, but in addition, 24DA6NT was formed. 24D (HA)6NT was detected only at the lowest TNT concentrations tested; and its occurrence was remarkably delayed versus the appearance of ADNTs (Fig. 3 compare c,d).

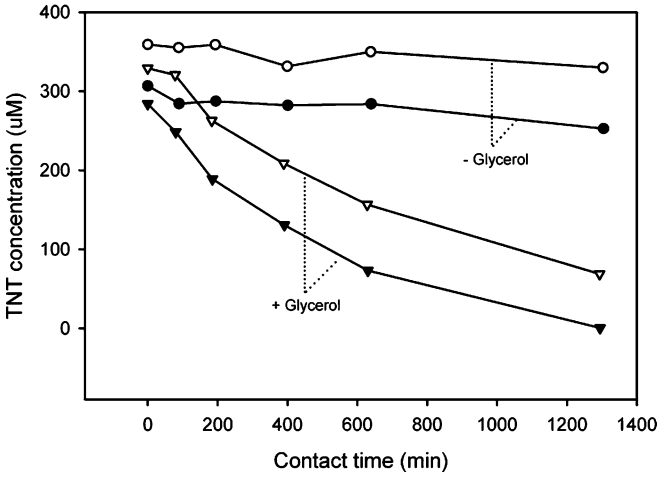


Fig. 2 Stimulatory effect of glycerol as an exogenous energy/electron source. High concentrations of TNT (280–360 μM) were contacted with a small amount of cells (OD_{600}=0.5) and replicates were supplemented with glycerol (+*Glycerol*, 20 mM) versus controls without glycerol (−*Glycerol*)

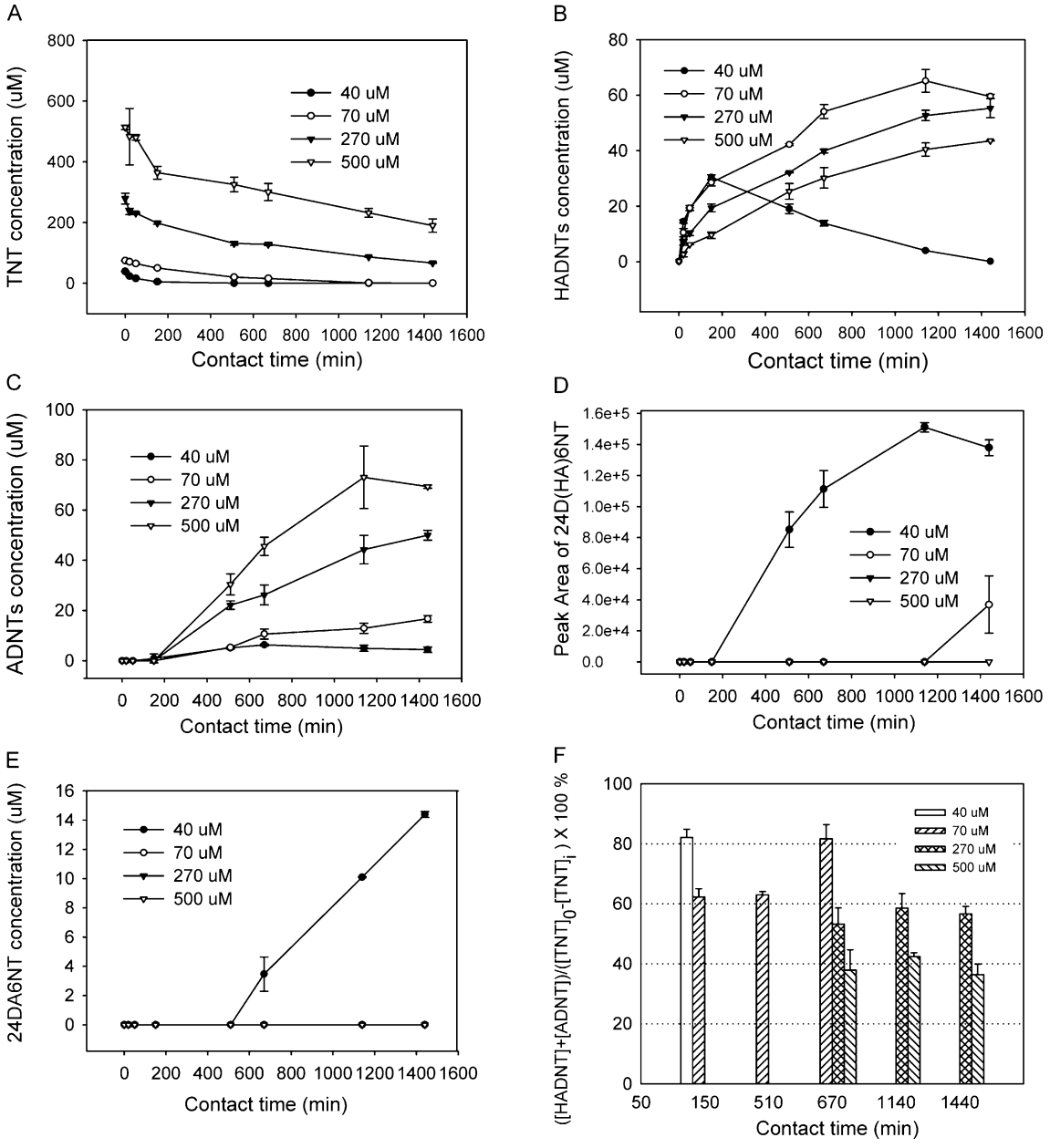


Fig. 3 **a–e** Time-course of TNT reductive intermediates at various initial TNT concentrations (C_i=40, 70, 270, 500 μM). Cell density OD_{600}=1.0. Cell protein concentration = 300 mg/l. *uM* Micromolar concentration. **f** Recovery of TNT over contact time at various initial TNT concentrations (C_i=40, 70, 270, 500 μM). Recovery is calculated as 100%×([HADNT] + [ADNT])/($[TNT]_0$ − final [TNT])

24DA6NT formation was clearly observed at the lowest TNT concentration tested: its first occurrence was after 600 min and it accounted for 14 μM after 1,500 min, when ADNT was approximately 4.4 μM. Traces of 24DA6NT (7 μM) were detected for C_i=70 μM (16 μM after 3,800 min), but no 24DA6NT was detected at the higher C_i tested (Fig. 3e).

Mass balance

Recovery was calculated as the ratio of the molar amount of HADNTs plus ADNTs formed over the molar amount of TNT transformed (Fig. 3f). At the lowest initial TNT concentrations (40 μM, 70 μM), HADNTs and ADNTs accounted for up to 80% of the transformed TNT. However, at higher initial TNT concentrations, mass recovery was significantly lower (60% at C_i =270 μM, 40% at C_i = 500 μM).

To examine the significance of TNT partitioning onto solid phases, cell pellets plus reaction glassware were extracted at the end of the reaction (24 h). Sorbed TNT concentration accounted for 0.6±0.24% and 1±0.13% of the original TNT for initial TNT concentrations of 270 μM and 500 μM, respectively. At lower initial TNT concentrations, no solid-phase-associated TNT was detected.

Discussion

Carefully controlled resting-cell experiments were performed with *E. coli* cultures pre-grown under aerobic conditions to defined cell densities. The resting-cell assays were performed in an anaerobic atmosphere to avoid competition of O_2 for cellular reducing equivalents and/or promotion of O_2-catalyzed polymerization of reduction intermediates (Wang et al. 2003). As a result, no azoxy-dimers were detected in our experiments, while reasonable mass balances were obtained. HADNTs and ADNTs accounted for up to 80% of transformed TNT at initial concentrations of 40 μM and 70 μM, although lower recoveries were noted at C_i=270 μM and C_i=500 μM. These recoveries compare favorably with other controlled TNT reduction studies [e.g., 65% in enzymatic transformations of TNT by NAD(P)H/flavin mononucleotide (FMN) oxidoreductase (Riefler and Smets 2002), 80% in assays with *P. fluorescens* under nitrate-reducing conditions (Gilcrease and Murphy 1995)]. Abiotic experiments clearly revealed the need for a biocatalyst in TNT reduction and the necessity for a brief mixing period after TNT addition, before TNT concentrations attained a steady concentration (as observed by others; Lewis et al. 1996). The cell-associated TNT concentrations were insignificant, as revealed by analysis of acetonitrile extracts of cell pellet and the glass wall of the reaction tubes.

Based on the experiments performed, a pathway for reductive transformation of TNT by *E. coli* can be proposed (Fig. 4). This pathway shows many similarities and some deviations from known TNT reductive pathways (McCormick et al. 1976; Spain 1995). TNT is sequentially transformed to 4HA26DNT and 2HA46DNT (with 4HA26DNT as the major isomer), followed by formation of 24D(HA)6NT and ADNT isomers (4A26DNT, with sporadic detection of 2A46DNT), all of which—except 24D(HA)6NT—were detected in presence of residual TNT, indicating concomitant reduction. Formation of 2HA4A6NT from 24D(HA)6NT was observed in other systems (Fiorella and Spain 1997) and 24DA6NT was the most reduced transformation product detected. Few analytically detailed investigations on TNT reduction by *E. coli* exist, often only examining TNT disappearance (Fuller and Manning 1997) and making a rigorous comparison difficult (Ederer et al. 1997; Fuller and Manning 1997). However, anaerobically grown *E. coli* was reported to display simultaneous accumulation of 4A26DNT and 24DA6NT (Ederer et al. 1997), inconsistent with our observation, where 24DA6NT accumulation is clearly delayed from ADNT formation (Fig. 3). The formation of 24DA6NT as final compound is consistent with its formation by aerobically or anaerobically grown *E. coli* cells (Ederer et al. 1997; McCormick et al. 1976), although TAT has been detected under a hydrogen atmosphere with cell extracts of anaerobically grown *E. coli* cells. Conversion of 24DA6NT to 4-*N*-acetylamino-2-amino-6-nitrotoluene was reported for *P. fluorescens* under aerobic conditions (Gilcrease and Murphy 1995), but was not observed in our experiments.

24D(HA)6NT was identified before the formation of 24DA6NT; and the formation of 24D(HA)6NT seemed to be inhibited by TNT. 24D(HA)6NT has never been reported in previous *E. coli* studies (Ederer et al. 1997; McCormick et al. 1976), but 4HA26DNT has been observed under a hydrogen atmosphere with both aerobically and anaerobically grown *E. coli* (McCormick et al. 1976). Under anaerobic conditions with both cell extracts and whole cells of *Clostridium* spp, 24D(HA)6NT has been shown to undergo a Bamberger rearrangement to form 4-amino-6-hydroxylamino-3-methyl-2-nitrophenol (Hughes et al.

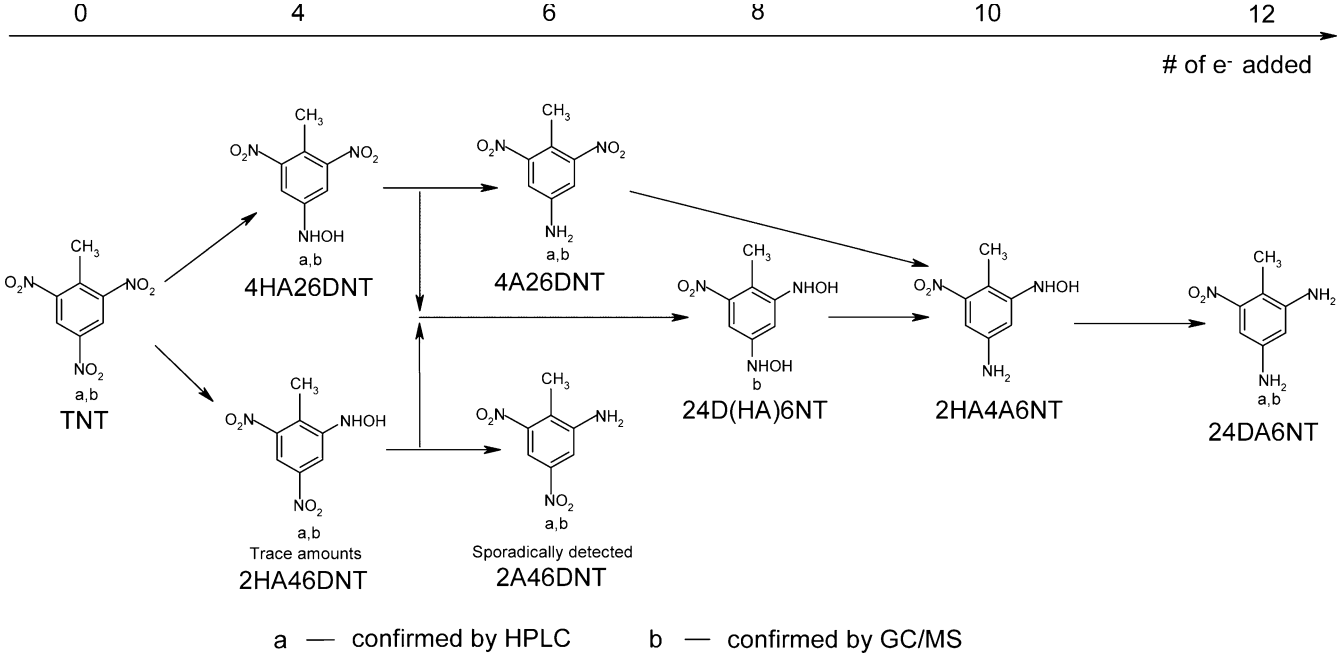


Fig. 4 Proposed reductive pathway for TNT transformation by *E. coli*

1998). HADNTs can also be subject to such Bamberger rearrangement to form their corresponding phenolamines (Hawari et al. 1999). These rearranged compounds have molecular masses identical to their parent compounds and should result in a second peak in the GC/MS with same derivatized molecular mass. Such peaks were not observed in our experiment, suggesting that the enzymatic activity for Bamberger rearrangement was absent in the tested *E. coli* strain. Nor were azoxytoluenes detected, confirming that oxygen-devoid incubation prevented their formation (Wang et al. 2003). The compounds indicated above accounted for all peaks detected in HPLC or GC/MS chromatograms.

Significant preference for reduction in the *para* versus the *ortho* position (≥approx. 10:1) was found, consistent with other bacterial and enzymatic TNT reduction studies (Riefler and Smets 2002), and likely due to the larger degree of polarization and protonation of the nitro substituent in the *para* position (Huang and Leszczynski 2002).

The detected pathway intermediates all reflect either addition of four electrons (hydroxylamino intermediates) or two electrons (amino substituents) from previously detected intermediates. Following the paradigm of two-electron reduction, this suggests that the nitroso intermediates are highly reactive and are rapidly reduced to hydroxylamino derivatives (Esteve-Nunez et al. 2001; Spain 1995); and this is consistent with many other studies on TNT or other nitroarene reduction, where nitroso derivatives are typically not detected (Fiorella and Spain 1997). The conversion of HADNT to 24D(HA)6NT that we infer has also been observed for the CO dehydrogenase from *C. thermoaceticum*, the Fe-only hydrogenase in *C. acetobutylicum*, and the nitrobenzene reductase from *P. pseudoalcaligenes* strain JS52 (Fiorella and Spain 1997). In the Enterobacteriaceae, several enzymes with nitroreductase activity have been characterized: they include the *nfsA*- and *nfsB*-encoded nitroreductases in *E. coli* (Rau and Stolz 2003) and the *nfsI* encoded nitroreductase in *Enterobacter cloacae* (Hannink et al. 2001). All enzymes are closely related: they are oxygen-insensitive type I nitroreductases and homodimeric flavoproteins containing FMN as cofactor, using NAD(P)H or NADH as electron donor, and performing two-electron reductions of nitro substituents (Bryant et al. 1991), although nitroso-intermediates are typically not detected (Koder and Miller 1998). The nitroreductase from *E. cloacae* (NR), isolated from a weapons storage dump, was found to reduce TNT (Bryant et al. 1991). A direct implication of TNT reduction by the *nfsA*- or *nfsB*-encoded enzymes in *Escherichia coli* has not been demonstrated, but is expected given the wide electron acceptor range of these enzymes (Carroll et al. 2002; Zenno et al. 1996).

Our experiments clearly indicate that the extent of TNT reduction is in large part controlled by the availability of reducing power, which can be modified by increasing the concentration of active cell mass in the experiments, or by supplementation of an exogenous electron donor (Fig. 2). While such observation is merely consistent with the fact that nitroreductases require and can be limited by intracellular reducing power [usually in the form of NAD(P)H; Riefler and Smets 2000], it suggests one mechanism to control the end-products in reductive TNT transformation. It should be noted that, in addition to reducing power limitation, toxicity (due to TNT or its transformation products; Riefler and Smets 2002) may in part contribute to an apparent effect of cell mass or exogenous carbon source concentration on reduction.

In this study, the pathway intermediates in reductive TNT transformation by *E. coli* have been identified through detailed HPLC and GC/MS analysis. Important factors that control the TNT transformation rate and extent have been preliminarily investigated, including the amount of biocatalyst and energy/reducing power. Future work is geared to establish the direct link between the *nfsA*- and *nfsB*-encoded enzymes and TNT reduction or to identify other responsible enzymes.

Acknowledgements This work was supported by an Award from the National Science Foundation to B.F.S. and T.K.W. (BES-0114126). We thank Dr. Joseph Hughes (Georgia Institute of Technology) for providing the dihydroxylaminonitrotoluene standard, Dr. Kenneth M. Noll (University of Connecticut) for discussions on *E. coli* physiology, and Dr. Glenn Johnson and Ms. Shirley F. Nishino (Air Force Research Laboratory) for sharing details on chemical analytical techniques. The experiments comply with the current laws of the United States in which the experiments were performed.

References

Boopathy R, Manning JF (1996) Characterization of partial anaerobic metabolic pathway for 2,4,6-trinitrotoluene degradation by a sulfate-reducing bacterial consortium. Can J Microbiol 42:1203–1208

Bryant C, Hubbard L, McElroy W (1991) Cloning, nucleotide sequence, and expression of the nitroreductase gene from *Enterobacter cloacae*. J Biol Chem 266:4126–4130

Carroll CC, Warnakulasuriyarachchi D, Nokhbeh MR, Lambert IB (2002) *Salmonella typhimurium* mutagenicity tester strains that overexpress oxygen-insensitive nitroreductases *nfsA* and *nfsB*. Mutat Res/Fundam Mol Mech Mutagenesis 501:79–98

Ederer MM, Lewis TA, Crawford RL (1997) 2,4,6-Trinitrotoluene (TNT) transformation by *clostridia* isolated from a munition-fed bioreactor: comparison with non-adapted bacteria. J Ind Microbiol Biotechnol 18:82–88

Esteve-Nunez A, Caballero A, Ramos JL (2001) Biological degradation of 2,4,6-trinitrotoluene. Microbiol Mol Biol Rev 65: 335–352

Fiorella P, Spain J (1997) Transformation of 2,4,6-trinitrotoluene by *Pseudomonas pseudoalcaligenes* JS52. Appl Environ Microbiol 63:2007–2015

French CE, Nicklin S, Bruce NC (1998) Aerobic degradation of 2,4,6-trinitrotoluene by *Enterobacter cloacae* PB2 and by pentaerythritol tetranitrate reductase. Appl Environ Microbiol 64: 2864–2868

Fuller ME, Manning JF (1997) Aerobic gram-positive and gram-negative bacteria exhibit differential sensitivity to and transformation of 2,4,6-trinitrotoluene (TNT). Curr Microbiol 35: 77–83

Gilcrease PC, Murphy VG (1995) Bioconversion of 2,4-diamino-6-nitrotoluene to a novel metabolite under anoxic and aerobic conditions. Appl Environ Microbiol 61:4209–4214

Haidour A, Ramos JL (1996) Identification of products resulting from the biological reduction of 2,4,6-trinitrotoluene, 2,4-dinitrotoluene, and 2,6-dinitrotoluene by *Pseudomonas* sp. Environ Sci Technol 30:2365–2370

Hannink N, Rosser SJ, French CE, Basran A, Murray JAH, Nicklin S, Bruce NC (2001) Phytodetoxification of TNT by transgenic plants expressing a bacterial nitroreductase. Nat Biotechnol 19: 1168–1172

Hawari J, Halasz A, Beaudet S, Paquet L, Ampleman G, Thiboutot S (1999) Biotransformation of 2,4,6-trinitrotoluene with *Phanerochaete chrysosporium* in agitated cultures at pH 4.5. Appl Environ Microbiol 65:2977–2986

Honeycutt ME, Jarvis AS, McFarland VA (1996) Cytotoxicity and mutagenicity of 2,4,6-trinitrotoluene and its metabolites. Ecotoxicol Environ Saf 35:282–287

Huang M-J, Leszczynski J (2002) The mechanism of the radical-anion reduction of 2,4,6-trinitrotoluene: a theoretical insight. J Mol Struct THEOCHEM 592:105–113

Hughes JB, Wang C, Yesland K, Richardson A, Bhadra R, Bennett G, Rudolph F (1998) Bamberger rearrangement during TNT metabolism by *Clostridium acetobutylicum*. Environ Sci Technol 32:494–500

Johnson GR, Smets BF, Spain JC (2001) Oxidative transformation of aminodinitrotoluene isomers by multicomponent dioxygenases. Appl Environ Microbiol 67:5460–5466

Koder RL, Miller AF (1998) Steady-state kinetic mechanism, stereospecificity, substrate and inhibitor specificity of *Enterobacter cloacae* nitroreductase. Biochem Biophys Acta 1387: 395–405

Lewis T, Goszczynski S, Crawford R, Korus R, Admassu W (1996) Products of anaerobic 2,4,6-trinitrotoluene (TNT) transformation by *Clostridium bifermentans*. Appl Environ Microbiol 62:4669–4674

McCormick NG, Feeherry FE, Levinson HS (1976) Microbial transformation of 2,4,6-trinitrotoluene and other nitroaromatic compounds. Appl Environ Microbiol 31:949–958

Montgomery HAC (1961) The determination of nitrite in water. Analyst 86:414–416

Pasti-Grigsby MB, Lewis TA, Crawford DL, Crawford RL (1996) Transformation of 2,4,6-trinitrotoluene (TNT) by actinomycetes isolated from TNT-contaminated and uncontaminated environments. Appl Environ Microbiol 62:1120–1123

Rau J, Stolz A (2003) Oxygen-insensitive nitroreductases NfsA and NfsB of *Escherichia coli* function under anaerobic conditions as lawsone-dependent Azo reductases. Appl Environ Microbiol 69:3448–3455

Riefler RG, Smets BF (2000) Enzymatic reduction of 2,4,6-trinitrotoluene and related nitroarenes: kinetics linked to one-electron redox potentials. Environ Sci Technol 34:3900–3906

Riefler RG, Smets BF (2002) NAD(P)H:flavin mononucleotide oxidoreductase inactivation during 2,4,6-trinitrotoluene reduction. Appl Environ Microbiol 68:1690–1696

Sax NI, Lewis RJ (1987) Hawley's condensed chemical dictionary, 11th edn. Van Nostrand, New York

Spain JC (1995) Biodegradation of nitroaromatic compounds. Annu Rev Microbiol 49:523–555

Spain JC, Hughes JB, Knackmuss H-J (2000) Perspectives of bioelimination of polynitroaromatic compounds. Biodegradation of nitroaromatic compounds and explosives. Lewis, New York

Spanggord RJ, Spain JC, Nishino SF, Mortelmans KE (1991) Biodegradation of 2,4-dinitrotoluene by a *Pseudomonas* sp. Appl Environ Microbiol 57:3200–3205

Wang C, Lyon DY, Hughes JB, Bennett GN (2003) Role of hydroxylamine intermediates in the phytotransformation of 2,4,6-trinitrotoluene by *Myriophyllum aquaticum*. Environ Sci Technol 37:3595–3600

Watrous MM, Clark S, Kutty R, Huang S, Rudolph FB, Hughes JB, Bennett GN (2003) 2,4,6-Trinitrotoluene reduction by an Fe-only hydrogenase in *Clostridium acetobutylicum*. Appl Environ Microbiol 69:1542–1547

Zenno S, Koike H, Kumar A, Jayaraman R, Tanokura M, Saigo K (1996) Biochemical characterization of NfsA, the *Escherichia coli* major nitroreductase exhibiting a high amino acid sequence homology to Frp, a *Vibrio harveyi* flavin oxidoreductase. J Bacteriol 178:4508–4514