

TNT biotransformation: when chemistry confronts mineralization

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Abstract Our understanding of the genetics and biochemistry of microbial 2,4,6-trinitrotoluene (TNT) biotransformation has advanced significantly during the past 10 years, and biotreatment technologies have developed. In this review, we summarize this new knowledge. A number of enzyme classes involved in TNT biotransformation include the type I nitroreductases, the old yellow enzyme family, a respiration-associated nitroreductase, and possibly ring hydroxylating dioxygenases. Several strains harbor dual pathways: nitroreduction (reduction of the nitro group in TNT to a hydroxylamino and/or amino group) and denitration (reduction of the aromatic ring of TNT to Meisenheimer complexes with nitrite release). TNT can serve as a nitrogen source for some strains, and the postulated mechanism involves ammonia release from hydroxylamino intermediates. Field biotreatment technologies indicate that both stimulation of microbial nitroreduction and phytoremediation result in significant and permanent immobilization of TNT via its metabolites. While the possibility for TNT mineralization

was rekindled with the discovery of TNT denitration and oxygenolytic and respiration-associated pathways, further characterization of responsible enzymes and their reaction mechanisms are required.

Keywords TNT · Biotransformation · Nitroreductase · Denitration · Old yellow enzyme · Phytoremediation

Introduction

2,4,6-Trinitrotoluene (TNT) has dominated the military production industry since 1902 and reached its peak during the two World Wars. Because of its massive production and use, many sites have become severely contaminated with this explosive and related compounds (Hampton and Sisk 1997). Growing concern about the health and ecological threats posed by explosives has led to extensive studies of their toxicology. TNT poses a high intrinsic toxic potential on the ecosystem, including humans and aquatic and terrestrial organisms (Talmage et al. 1999; Dodard et al. 2003; Robidoux et al. 2003; Lachance et al. 2004; Sarlauskas et al. 2004), and is listed as class C potential human carcinogen by the US Environmental Protection Agency. TNT toxicity in mammals is contingent on bioreductive activation to toxic hydroxylamino and amino metabolites (Leung et al. 1995; Sarlauskas et al. 2004), with formation of reactive oxygen species through redox cycling (Kumagai et al. 2000). Several epidemiological studies and animal experiments have shown that TNT induces reproductive toxicity through oxidative DNA damage mediated by its metabolites (Homma-Takeda et al. 2002).

While a solid understanding of the fate of TNT and its transformation products in microbially active systems existed 10 years ago, the last decade has witnessed a significant

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expansion of our knowledge on TNT biotransformation at the genetic and biochemical level and has seen the development and verification of field biotechnologies. In this review, therefore, we summarize new knowledge regarding genes, enzymes, and pathways involved in microbial TNT transformation (summarized in Fig. 1), while new findings on bioremediation technologies are also incorporated. For a more complete or historic view on TNT biodegradation/mineralization, the reader is referred to excellent works by Hawari et al. (2000), Esteve-Nuñez et al. (2001), Ramos et al. (2005), or Symons and Bruce (2006).

Enzymatic Transformation of TNT

Nitroreductases

A chief class of enzymes involved in TNT biotransformation are the nitroreductases (Fig. 1, B). This family of enzymes reduces a wide range of nitroaromatic compounds such as nitrofurazones, nitroarenes, nitrophenols, nitrobenzenes, and explosives including TNT, hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX), and glycerol trinitrate. While one historic singular publication anecdotes the existence of an oxygen sensitive nitroreductase (called type II nitroreductase) (Peterson et al. 1979; Bryant et al. 1981), all nitroreductases subject to the current study are oxygen insensitive (called type I nitroreductase). Nitroreductases contain flavin mononucleotide (FMN) as the cofactor, use NAD(P)H and/or NADH as the electron donor, and perform sequential two-electron reductions of nitro substituents (Bryant and DeLuca 1991; Bryant et al. 1991), although the nitroso intermediates are typically not detected (Koder and Miller 1998). Nitroreductases have recently been found across the bacterial domain, which include NfsA and NfsB in *Escherichia coli* (Zenno et al. 1996a,b; Paterson et al. 2002; Rau and Stolz 2003), SnrA and Cnr in *Salmonella enterica* serovar Typhimurium (Watanabe et al. 1990; Nokhbeh et al. 2002), NfsI in *Enterobacter cloacae* (Bryant and DeLuca 1991; Hannink et al. 2001; Haynes et al. 2002), RdxA in *Helicobacter pylori* (Goodwin et al. 1998), flavin reductase P in *Vibrio Harveyi* (Lei et al. 1994), Frase I in *Vibrio fisherii* (Zenno et al. 1994; Riefler and Smets 2002), NADH oxidase in *Thermus thermophilus* (Park et al. 1992), nitrobenzene nitroreductase in *Pseudomonas pseudoalcaligenes* (Somerville et al. 1995), PnrA and PnrB in *Pseudomonas putida* (Park and Kim 2000; Caballero et al. 2005b), and NitA and NitB in *Clostridium acetobutylicum* (Kutty and Bennett 2005). While *nfsA* is part of the *sox* regulon in *E. coli* and suggests its involvement in response to oxidative stress (Paterson et al. 2002), the actual physiological role of the nitroreductases in other strains is essentially unknown, except that *snrA* is also

inducible by paraquat (Nokhbeh et al. 2002). These nitroreductases consistently occur as pairs, which each member belonging to subgroups with very high phylogenetic relatedness across the examined strains (Fig. 2).

Although *pnrA* is constitutively expressed in *P. putida* JLR11 (Caballero et al. 2005b), expression of NitA and NitB is induced by the presence of TNT (Kutty and Bennett 2005). While these enzymes have a broad substrate range, PnrA and NitA/B seem to have the widest substrate range, including even di- and mononitrotoluenes (Caballero et al. 2005b; Kutty and Bennett 2005).

It has become increasingly apparent that nitroreductases reduce nitro to hydroxylamino functional groups and not beyond, even in the presence of excess reducing power (Riefler and Smets 2002; Watrous et al. 2003; Caballero et al. 2005b; Kutty and Bennett 2005). On the other hand, in whole-cell assays, reduction typically goes to the level of amino groups (see Table 1). In addition, the common observation that TNT is reduced faster than di- and mononitrotoluenes or reduced transformation products (Caballero et al. 2005b) can be explained well by the one-electron redox potential of these substrates (Riefler and Smets 2000).

Old yellow enzyme family

Another significant family of enzymes that shares the type I nitroreductase activity (i.e., nitro to amino reduction) but has no real homology is called the old yellow enzyme (OYE) family, named after the first enzyme shown to contain a bound flavin (FMN). XenA and XenB from *Pseudomonas* sp., pentaerythritol tetranitrate (PETN) reductase from *E. cloacae*, *N*-ethylmaleimide reductase (NemA) from *E. coli*, morphinone reductase from *P. putida* M10, as well as OYE from *Saccharomyces cerevisiae* belong to this class (Pak et al. 2000; Khan et al. 2002; Orville et al. 2004a,b; Williams et al. 2004). YqjM from *Bacillus subtilis*, a yeast OYE homologue, shares many characteristic biochemical properties: It is able to transform TNT (Fitzpatrick et al. 2003) and appears inducible by TNT.

Some of these enzymes—in addition to being able to transform TNT via nitroreduction—are able to liberate nitrite from TNT by denitration (note that OYE itself has no denitration reactivity against TNT [Williams et al. 2004]; Fig 1, A). The nitroreduction pathway is catalyzed by all the enzymes, while denitration is only catalyzed by PETN reductase, XenB, and NemA via the formation of TNT hydride–Meisenheimer complex (H^-TNT) and TNT dihydride–Meisenheimer complex ($2H^-TNT$; Meah et al. 2001; Williams et al. 2004). Although $2H^-TNT$ has been reported as unstable (Pak et al. 2000), it has also been shown to be enzymatically transformed by OYE enzymes with subsequent or concomitant nitrite release (Williams et al. 2004).

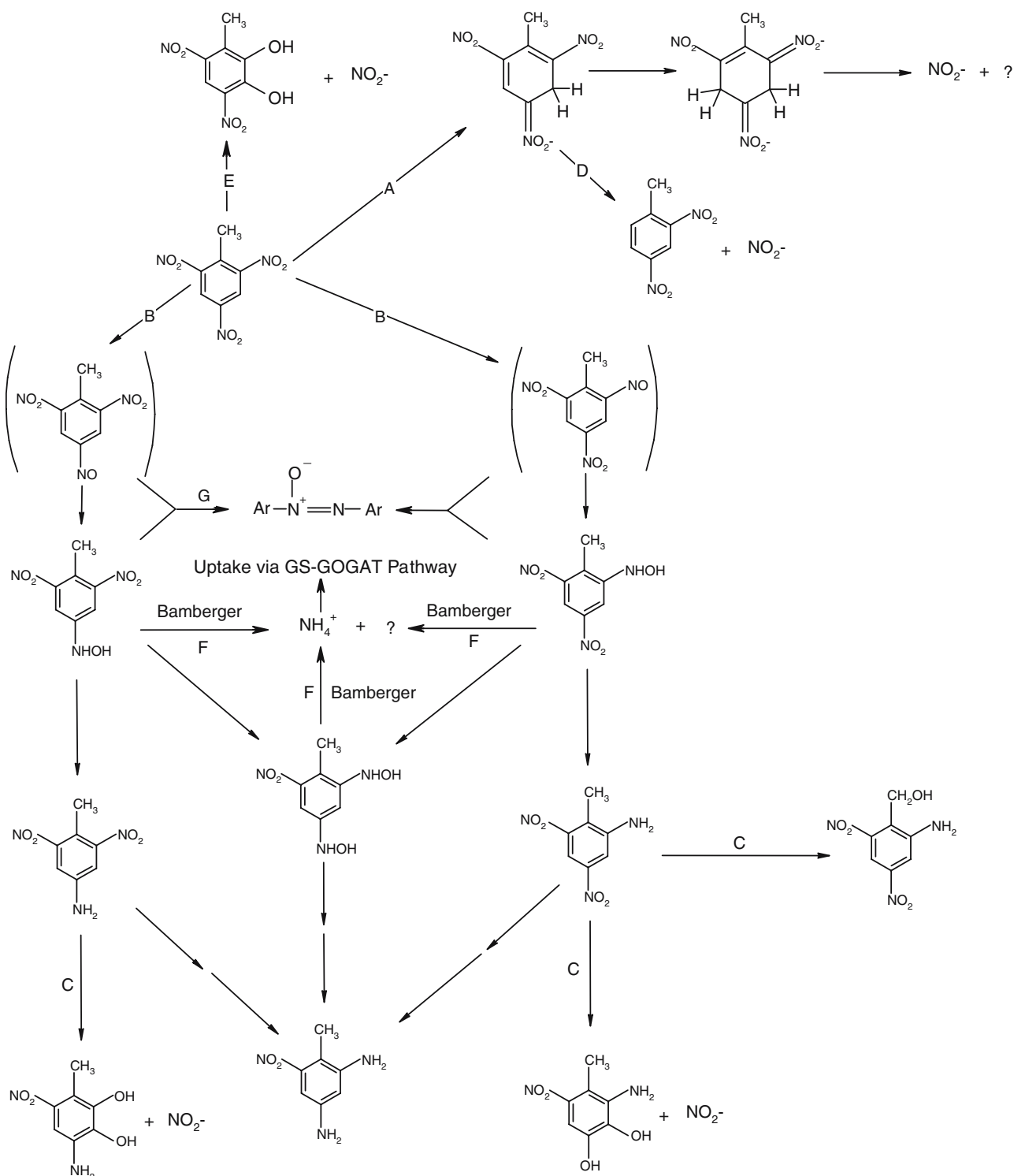


Fig. 1 Mechanisms of TNT biodegradation pathways. **A**, Reduction of aromatic ring via a Meisenheimer complex with the release of nitrite (Williams et al. 2004; Ramos et al. 2005). **B**, Reduction of the nitro group of TNT to a series of reduced intermediates (Esteve-Nuñez et al. 2001; Yin et al. 2004; Yin et al. 2005). **C**, Aminodinitrotoluenes are oxidized by dioxygenases to form aminomethylnitro catechol releasing nitrite or are hydroxylated at the methyl group to form aminodinitrobenzyl alcohol (Johnson et al. 2001; Keenan et al. 2005; Keenan and Wood 2006). **D**, The hydride-Meisenheimer complex

(H-TNT) is subsequently denitrated to 2,4-dinitrotoluene (2,4-DNT; Jain et al. 2004). **E**, An oxygenolytic pathway results in the production of 3-methyl-4,6-dinitro catechol through removal of a nitro group (Tront and Hughes 2005). **F**, Bamberger-like rearrangement of hydroxylamino intermediates is followed by ammonium release and subsequent uptake via the glutamine synthetase-glutamate synthase (GS-GOGAT) pathway (Caballero et al. 2005a). **G**, Formation of azoxytetranitrotoluenes dimers from the reaction of nitroso and hydroxylamino intermediates (Haidour and Ramos 1996)

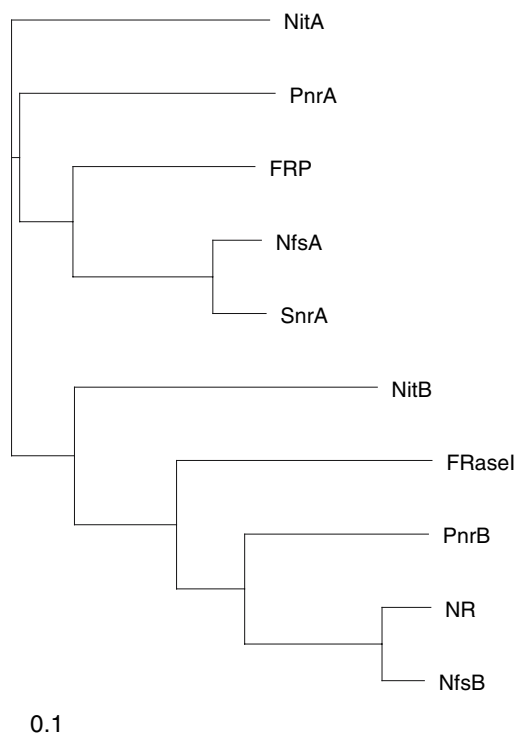


Fig. 2 Phylogenetic relationship of true Type I nitroreductases. The sources of the sequences were as follows: NfsA (GenBank P17117) and NfsB (D25414) from *E. coli*, FRP from *V. harvey* (GenBank Q56691), FRaseI from *V. fischerii* (P46072), SnrA from *S. typhimurium* (AAD18027), NR from *E. cloacae* (Q01234), PnrA (Q93AS3) and PnrB (Q8KRI2) from *Pseudomonas* sp. JLR11, and NitA and NitB from *C. acetobutylicum*. Alignments were made in CLUSTALW, and the distance method based on percent identity was used to infer the tree

Comparison of the functional properties of OYE family members has identified His/Asn 184 (of PETN reductase) as the key substrate-binding residue that might determine the outcome of the initial reduction of TNT (Williams et al. 2004; nitroreduction vs direct hydride addition), and evidence for direct hydride transfer from the reduced enzyme-bound flavin to the carbon skeleton was inferred (Khan et al. 2002).

The exact physiological role of these OYE enzymes is still unclear, but they are probably part of a general stress response system (e.g., induction upon oxidative stress) and may maintain proper intracellular redox state, by transferring electrons from pyrimidine nucleotides to suitable oxidants such as quinones (Fitzpatrick et al. 2003).

Respiration-associated nitroreductase

Although TNT has classically been considered as a possible nitrogen source, in *P. putida* JLR11, it can serve the unexpected role of terminal electron acceptor. *P. putida* JLR11 contains a membrane-associated respiratory nitroreductase that confers this strain the capacity to use nitro groups from TNT as the sole electron acceptor while oxidizing the electron donor acetate under anaerobic

conditions. This novel respiratory process was supported by the measurement of proton translocation and ATP synthesis coupled to the oxidation of acetate or H_2 concomitant with the reduction of TNT to 4-amino-2,6-dinitrotoluene (4ADNT) in whole cells or membrane vesicles, respectively (Esteve-Nuñez et al. 2000).

Ring hydroxylating dioxygenases

Because TNT is easily reduced to an isomeric mixture of 2-amino-4,6-dinitrotoluene (2ADNT) and 4ADNT under environmental conditions (Spain et al. 2000), a number of studies have been focused on the possible oxygenolytic attack of the aminodinitrotoluene (ADNT) isomers (Fig. 1, C). Recombinant *E. coli* expressing the 2,4-dinitrotoluene (DNT) dioxygenase (R34DDO) from *Burkholderia cepacia* R34 or *Burkholderia* sp. strain DNT are capable of oxidizing 2ADNT at the 3,4 position releasing nitrite to form 3-amino-4-methyl-5-nitrocatechol, while also hydroxylating the methyl group to form 2-amino-4,6-dinitrobenzyl alcohol (Johnson et al. 2001). The recombinant nitrobenzene dioxygenase (NBDO) from *Comamonas* sp. strain JS765 can oxidize 4ADNT at the 2,3 position releasing nitrite and to generate 3-amino-6-methyl-5-nitrocatechol (Johnson et al. 2001). Combining the terminal oxygenases of NBDO and R34DDO has yielded a so-called orthric hybrid dioxygenase that can even mediate the simultaneous oxidation of 2ADNT and 4ADNT yielding the expected catechols as well as the benzylalcohol, reported above (Keenan and Wood 2006). Despite the promises of the oxygenolytic cleavage of the aromatic ring, the activity of these dioxygenases seems pretty low (generally less than $0.5 \text{ nmol product min}^{-1} \text{ mg protein}^{-1}$), especially for the more recalcitrant 4ADNT, and whether the formed aminomethylnitrocatechols can be enzymatically transformed or remain dead-end products is unsolved.

Other enzymes

Fe-only hydrogenase

The Fe-only hydrogenase from *C. acetobutylicum*, typically associated with H_2 production, catalyzes a H_2 -dependent reduction of TNT to 2-hydroxylamino-4,6-dinitrotoluene (2HADNT) and 4-hydroxylamino-2,6-dinitrotoluene (4HADNT) and subsequent reduction of these compounds to 2,4-dihydroxylamino-6-nitrotoluene (24D(HA)6NT) during acidogenic phase (Watrous et al. 2003). Inhibition studies using antibodies have revealed core parts of the enzyme necessary for TNT reduction, while [4Fe-4S] centers have been inferred as essential from Cu^{2+} inhibition (Kutty and Bennett 2006).

Table 1 Recently reported TNT transformation products by various strains

Strain	Aerobic/ anaerobic	Intermediates	References
<i>Escherichia coli</i>	Aerobic	2HADNT, 4HADNT, 2ADNT, 4ADNT 24D(HA)6NT, 4DA6NT, NO ₂ ⁻	Yin et al. 2004; Stenuit et al. 2006
<i>Raoultella terrigena</i> HB	Aerobic	2ADNT, 4ADNT, 24DA6NT, azoxy-dimers, NO ₂ ⁻	Claus et al. 2007
<i>Pseudomonas aeruginosa</i> MX	Aerobic	2HADNT, 4HADNT, 2ADNT, 2,2'-azoxy-dimers, NO ₂ ⁻ , 2,4DNT, 4NT, 3,5DNA.	Oh et al. 2003
<i>Yarrowia lipolytica</i> NCIM 3589	Aerobic	H ⁻ -TNT, 24DNT, ADNT	Jain et al. 2004
<i>Pseudomonas putida</i> KP-T202	Aerobic	2ADNT, 4ADNT, 24DNT, 26DNT	Park et al. 2003b
<i>Irpex lacteus</i>	Aerobic	H ⁻ -TNT, 2HADNT, 4HADNT, 2ADNT, 4ADNT	Kim and Song 2003
<i>Saccharomyces</i> sp. ZS-A1	Aerobic	H ⁻ -TNT, 2HADNT, 4HADNT, 2ADNT, 4ADNT	Zaripov et al. 2002
<i>Candida</i> sp. AN-L14	Aerobic	H ⁻ -TNT, 2HADNT, 4HADNT, 2ADNT, 4ADNT	Zaripov et al. 2002
<i>Candida</i> sp. AN-L13	Aerobic	H ⁻ -TNT	Zaripov et al. 2002
<i>Fusarium oxysporum</i>	Anaerobic	2ADNT, 4ADNT, 24DA6NT	Hoehamer et al. 2006b
<i>Cellulomonas</i> sp. ES6	Anaerobic	4HADNT, 2HADNT, 24D(HA)6NT, 4ADNT	Borch et al. 2005
<i>Klebsiella</i> sp. C1	Anaerobic	HADNTs, ADNTs, DANts, DNTs, NO ₂ ⁻	Kim et al. 2002

Nitroreductase I

Three NAD(P)H-dependent nitroreductases, without significant sequence homology to the nitroreductase or OYE family discussed above, have been detected in *Klebsiella* sp. C1 (Kim and Song 2005). The enzyme with highest activity, nitroreductase I, has FMN as its cofactor, catalyzes the two-electron reduction of several nitroaromatic compounds, and produces 2ADNT and 2,2',6,6'-tetranitro-4,4'-azoxytoluene from TNT. Hence, unlike other nitroreductases, it transforms hydroxylaminodinitrotoluenes (HADNTs) into ADNT, with preference for reduction in the ortho position. Even 2,4-DNT was subject to nitroreduction in the ortho position only to yield 2-hydroxylamino-4-nitrotoluene.

In summary, a growing number of bacterial nitroreductases have been isolated, which transform TNT to HADNTs through direct reduction of the nitro group. On the other hand, OYE-type enzymes appear less ubiquitous, and these can mediate the same reaction but can also attack the aromatic ring directly to form a dihydride–Meseinheimer complex of TNT and yield denitration products. Finally, one case of respiratory TNT use has been documented. The difference between reaction products observed in pure enzyme vs whole-cell transformation experiments suggests the involvement of yet another group of enzymes that can transform TNT and its reduction products.

Whole-cell transformation of TNT

Bacteria

TNT has been classically reported as transformed by bacteria through partial reduction of nitro groups leading

to ADNT (Yin et al. 2004; Yin et al. 2005; see Esteve-Núñez et al. 2001 for an extensive review) but, occasionally, also through full reduction to triaminotoluene when the redox potential becomes low enough (Preuss et al. 1993; Lewis et al. 1997). In addition, it has been known for more than a decade that the aromatic nucleus of TNT can be microbially reduced to a hydride–Meisenheimer complex (Vorbeck et al. 1994; Haidour and Ramos 1996; French et al. 1998).

While the involvement of bacteria is typically direct, it may also be indirect. For example, the fermentative bacterium *Cellulomonas* sp. strain ES6 reduces TNT faster and more complete (e.g., to diaminonitrotoluenes) when external electron shuttles (like anthraquinone-2,6-disulfonate or Fe(II)/ferrihydrite) are present (Borch et al. 2005).

Recently, the use of TNT as a true nitrogen source has been documented with both mixed (Popescu et al. 2003; Robertson and Jjemba 2005) and pure cultures. *P. putida* JLR11 uses TNT as a sole nitrogen source, with the release of nitrite and/or ammonium, and with involvement of the glutamine synthetase–glutamate synthase pathway (Caballero et al. 2005a,b; Caballero and Ramos 2006; Stenuit et al. 2006). While nitrite release is postulated to occur via one or more denitrases, ammonia release is postulated to occur after reduction of one of the nitro groups to a hydroxylamino moiety mediated by PnrA, followed by a Bamberger-like rearrangement, wherein the hydroxylamino functional group is rearranged to yield vicinal amino and hydroxyl substituents (Hughes et al. 1998; Caballero et al. 2005a; Caballero and Ramos 2006; Fig. 1, F). In addition, aerobic growth of *E. coli* EPI300 on TNT as a sole nitrogen source has also been reported, with simultaneous release of significant amounts of nitrite (up to 0.5 mole/mole TNT; Stenuit et al. 2006). Significant denitration was even observed (and favored over nitroreduction) in Luria–Bertani pregrown

resting cell assays (Stenuit et al. 2006), inconsistent with our own observations on TNT transformation with *E. coli* in resting cell (Yin et al. 2004) or cell extract assays (Yin and Smets, unpublished results) where rapid and near stoichiometric nitroreduction was observed. While the mechanism of nitrogen release/assimilation and denitration by *E. coli* were not identified, they were the postulated results of *E. coli*'s nitroreductases (NfsA, NfsB) and OYE NemaA, respectively. Others have noted that the denitration pathway by *E. coli* K12 is only induced above a threshold TNT concentration (Kurinenko et al. 2005). A similar existence of simultaneous TNT denitration and nitroreduction was also measured in *Klebsiella* sp. strain C1 (Kim et al. 2002), where also traces of DNT were reported as a metabolite!

Denitration pathways are intuitively interesting, as they might lead to the production of sterically simpler compounds (e.g. 2,4-dinitrotoluene [24DNT]), which may be subject to further enzymatic transformation. However, this is not born out by the experimental evidence: In studies observing TNT denitration, DNT is rarely observed (Martin et al. 1997; Kim and Song 2000; Kim et al. 2002; Oh et al. 2003), mass balances are generally poor, true mineralization is not detectable or insignificant, denitration is concomitant with nitroreduction (Esteve-Núñez and Ramos 1998; Williams et al. 2004; Stenuit et al. 2006), and not all denitrated products are accounted for, although dimeric metabolites are recurrent observations (Williams et al. 2004; Stenuit et al. 2006).

While emphasis in bacterial degradation is on reductive TNT transformations, the possibility of an oxygenolytic pathway was recently rekindled by the detection of 3-methyl-4,6-dinitrocatechol as well as significant nitrite release during aerobic TNT transformations by a mixed culture maintained on a mixture of TNT and 2,4-DNT as sole C and N sources (Tront and Hughes 2005). Stable isotope patterns confirmed that the catechol is derived from TNT, and it is potentially the result of a denitrating ring dihydroxylation of TNT. However, the actual detection of the catechol might suggest a dead-end pathway; the amount of mineralization observed was very small (<0.1% after prolonged incubations), and sustained growth of the culture with TNT only as the N source was not reported (Tront and Hughes 2005).

Yeast and fungi

Similar initial TNT transformation patterns are observed across fungi, although basidiomycetes, especially white rot fungi, display the capacity for significant (>20%) TNT mineralization, largely because of their ligninolytic systems (Nyanhongo et al. 2005). *Saccharomyces* sp. ZS-A1 reduces TNT to a mixture of HADNT isomers, with ADNT and HTNT as minor products. Conversely, *Candida* sp.

AN-L13 transforms TNT almost quantitatively into HTNT, while *Candida* sp. AN-L14 produces an equimolar mix of HADNT and HTNT (Zaripov et al. 2002). The marine yeast *Yarrowia lipolytica* NCIM 3589 reduces TNT to HTNT and significant amounts of the denitration product 24DNT (>0.25 M/M TNT) as well as ADNTs. Increasing amounts of reducing equivalents in the form of glucose enhanced reduction of the aromatic ring to 24DNT, although the reduction of the nitro groups to amino groups remained the dominant functional pathway (Jain et al. 2004) (Fig. 1, D).

Unlike other white rot fungi, who perform initial nitroreduction TNT, a denitration pathway has been found in *Irpex lacteus* with documented occurrence of HTNT as well as DNT as a metabolite (Kim and Song 2003). This fungus attains final mineralization of up to 30% possibly because denitrated metabolites are more amenable to oxidation by the lignolytic system.

Biotreatment technologies for TNT contaminated soils

Technologies include composting, bioslurry processes, and phytoremediation. Composting and bioslurry are based on the cometabolic nitroreduction of TNT by undefined microorganisms (Thorn et al. 2002) and can handle concentrations of up to 1 g TNT/kg soil in typical time frames of weeks to months (Park et al. 2003a). The final outcome of these processes is that hydroxylamine and amine groups of the nitroarene ring react with quinone and carbonyl functional groups of the soil humic fraction, giving rise to immobilized covalently bound TNT derivatives that are not bioavailable and thus exhibit decreased toxicity (Knicker et al. 2001; Lewis et al. 2004). Hence, this approach builds on the fact that reaction intermediates during nitroreduction have a high chemical reactivity, and even in laboratory research, mass balances often indicate very large degrees of immobilization or dimerization during TNT transformation (Fig. 1, G; Wang et al. 2003; Claus et al. 2007). For example, *Raoultella terrigena* strain HB rapidly converts 80–90% of soluble TNT (at 10 to 100 mg/L) to a cell-bound fraction, mainly as azoxy-dimers (Claus et al. 2007). Similarly, *Pseudomonas aeruginosa* MX accumulated 71% of the initial ¹⁴C-TNT in the cell pellet (primarily 2,2'-azoxytoluene), leaving 21% (primarily 2ADNT) in the supernatant (Oh et al. 2003), and *P. putida* JLR11 converts 45% of the initial TNT to cell-bound intermediates under anaerobic conditions (Esteve-Núñez and Ramos 1998). On the other hand, oxidative coupling of TNT and its metabolites to soil organic matter, mediated by oxidases (e.g., laccases and peroxidases) is also well established (Thiele et al. 2002; Wang et al. 2002). A purified laccase from a fungi isolate *Tremetes hirsuta* was recently reported

that catalyzes complete immobilization of HADNTs to humic monomers (Nyanhongo et al. 2006).

In a rigorous evaluation of several bioremediation processes, 67–93% of the originally added TNT radioactivity became unextractable. This value increased only marginally after subsequent physical, chemical, or biological mobilization attempts, and neither TNT nor transformation products were ever extracted (Weiss et al. 2004). In complementary studies, a strong reduction in the ecotoxicological effects were also measured (Frische 2003). Hence, bioremediation of TNT-contaminated soils, contingent on immobilization, seems to result in residues with low mobilization potential and, hence, low hazardous impact (Weiss et al. 2004).

Because the above microbe-catalyzed processes typically lead to little TNT mineralization (Fritsche et al. 2000; Weiss et al. 2004), other approaches have been taken, which involve chemical preoxidation (modified Fenton's reagent consisting of H_2O_2 in the presence of Fe^{2+}) after microbial degradation: The coupled abiotic–biotic process yielded significantly more mineralization (up to nearly 75%) than the abiotic-only treatment for soils spiked with up to 5 g/kg of TNT. (Schrader and Hess 2004).

Phytoremediation of TNT-contaminated environments remains attractive because it potentially offers a low-cost alternative to the more intrusive biotechnological approaches mentioned above. The effects of plants on TNT transformation may be indirect by enhancing microbial TNT nitroreduction through supply of carbonaceous matter via rhizodeposition (e.g., Chang et al. 2004) or direct. In the latter case, plants do not process TNT towards mineralization but towards immobilization via the so-called green liver mechanism (Hannink et al. 2002; Subramanian et al. 2006).

In the first step, after root uptake, TNT is subject to initial transformations with most of these being the typical nitroreduction products HADNTs and ADNTs (Wang et al. 2003; Hoehamer et al. 2006a). In the second step—at the level of HADNTs or ADNTs—conjugation occurs with possible involvement of enzymes like glycosyl transferases and glutathione-*S*-transferases (Bhadra et al. 1999; Mezzari et al. 2005; Vila et al. 2005; Subramanian et al. 2006). Finally, TNT metabolites are removed from the cytoplasmic phase and sequestered in the cell wall and other nonextractable fractions (Sens et al. 1999). The observation of a very low extractability of TNT from plant materials and preferred partition in root mass makes phytoremediation—using various plant types—a viable treatment option (Schoenmuth and Pestemer 2004; Yoon et al. 2006). These processes appear quite well conserved across the plant types studied, and the limitation with respect to TNT transformation appears not to be lack of enzymatic machinery but toxicity tolerance. As a result, the range of

TNT tolerance has been significantly extended by construction of transgenic tobacco plants that express bacterial nitroreductase (*nfs* from *E. cloacae*; Hannink et al. 2001) or OYE (*onr*) genes (French et al. 1999).

Summary and look ahead

In the penultimate decade, the TNT biotransformation field was occupied with studies describing the microbial potential and identifying the formed metabolites. In the last decade, this work has continued (the almost ubiquity of the nitroreductase pathway has been described), expanded (respiratory use of TNT has been discovered), and refined (improved radiometric and spectroscopic techniques have identified a larger suite of metabolites and have especially documented irreversible sequestration in many situations). In addition, biochemical work with isolated enzymes (e.g., several nitroreductases and enzymes of the OYE family) has identified reaction mechanisms, and genes have been assigned (Table 2). This has led to some novel technological developments (e.g., bacterial transgenes in plants).

It is now well known that microbial nitroreductase activity is widely present in the microbial world, and metabolites of the TNT nitroreduction pathway, while remaining carcinogenic and mutagenic, may be unavoidable. More efforts are needed in isolating bacteria or identifying new pathways to degrade those metabolites. One of the true bottlenecks in describing and controlling TNT metabolism by living cells is the high number and reactivity of the nitro group substituents, which generate an impressive number of metabolites coming from the same parental molecule: Transformation products with amino, hydroxylamino, phenol, methyl, and carboxylate substituents at the six positions of the ring can be found. Hence, the inherently high chemical reactivity of TNT may preclude several metabolic options.

Initially, it was found that most strains utilized only one initial enzymatic path for the degradation of TNT (Oh et al. 2003; Watrous et al. 2003; Borch et al. 2005; Hoehamer et al. 2006b; Claus et al. 2007); however, the simultaneous occurrence of nitroreduction (the sequential reduction of the nitro groups to hydroxylamine and amine derivatives) and denitration (the formation of a hydride–Meisenheimer complex by hydride addition to the aromatic ring) in several strains has been documented (Zaripov et al. 2002; Kim and Song 2003; Jain et al. 2004; Williams et al. 2004; Kurinenko et al. 2005). Whether this denitration pathway is equally ubiquitous and whether it may provide a route for TNT transformation to more easily biodegradable products remains an open question. Indeed, essentially nothing is known about the reactions that lead to the removal of the nitro groups by denitrases. Further characterization of

Table 2 Recently reported TNT transformation products by various isolated enzymes

Strain	Enzyme	Aerobic/anaerobic	Intermediates	References
<i>E. cloacae</i> PB2	PETN reductase	Aerobic	2HADNT, 4HADNT 2ADNT, 4ADNT 24D(HA)6NT, H ⁻ -TNT 2H ⁻ -TNT	Williams et al. 2004
<i>E. cloacae</i> type strain	PETN reductase	Aerobic	2HADNT, 4HADNT 2ADNT, 4ADNT 24D(HA)6NT, H ⁻ -TNT 2H ⁻ -TNT	Williams et al. 2004
<i>E. coli</i> JM109	NEM reductase	Aerobic	2HADNT, 4HADNT 2ADNT, 4ADNT 24D(HA)6NT, H ⁻ -TNT 2H ⁻ -TNT	Williams et al. 2004
<i>P. putida</i> M10	Morphinone reductase	Aerobic	2HADNT, 4HADNT 2ADNT, 4ADNT 24D(HA)6NT	Williams et al. 2004
<i>S. carlsbergensis</i>	OYE	Aerobic	2HADNT, 4HADNT 2ADNT, 4ADNT 24D(HA)6NT	Williams et al. 2004
<i>C. acetobutylicum</i>	Fe-only hydrogenase	Anaerobic	2HADNT, 4HADNT 24D(HA)6NT	Watrous et al. 2003
<i>P. putida</i> JLR11	Nitroreductase PnrA	Anaerobic	4HADNT	Caballero et al. 2005b
<i>V. fischeri</i>	NAD(P)H: FMN oxidoreductase	Anaerobic	2HADNT, 4HADNT	Riefler and Smets 2002

denitrases and their interactions with TNT will help in understanding these reaction mechanisms. Are dimeric metabolites necessary products in the denitration process, or can this metabolism be steered to structurally simpler compounds? A similarly interesting metabolism that requires further metabolic exploration is nitroreduction of TNT followed by use of the hydroxylamino-transformation products as a sole N source. While a Bamberger rearrangement has been speculated, nothing is known about the enzymes involved in the ultimate removal of the N atom from the aromatic nucleus.

Microbial mineralization of TNT remains an unrequited aspiration: A few pure enzyme studies have shown the capacity for oxygenolytic transformation of ADNTs to aminomethylnitrocatechols with the release of nitrite (Johnson et al. 2001; Keenan and Wood 2006), but no evidence of transformation beyond this point has been obtained. Mineralization, as inferred from CO₂ evolution using ¹⁴C-labeled TNT remains typically near detection limits. However, should TNT mineralization remain the target, or is it just a dogmatic fixation of microbiologists? Certainly, new molecular approaches like metagenomics, the culture-independent genomic analysis of entire microbial communities (Schloss and Handelsman 2003), will evaluate a much higher number of microbial biotransformations than classical enrichment procedures and might yield an unpredicted and novel set of genes encoding

enzymes that transform—or can be optimized for this function via protein engineering—TNT intermediates allowing a complete biodegradation of the original xenobiotic. However, on the other hand, full-scale biotechnological approaches based on stimulating nitroreduction in an environmental matrix, as well as phytoremediation, both reveal that TNT can be effectively removed from the environment by metabolite sequestration into nonmobile phases. It seems then that, from a technological point, we have come very close to acceptable solutions.

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