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Biological Remediation of Explosive Residues

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Dedicated to parents in heavenly abode

Preface

Cyclic nitramine explosives RDX, HMX, and CL-20 are commonly synthesized as most widespread conventional explosives. Their use in military munitions largely for the protection of national boundaries and mining operations, has resulted in widespread contamination of soil and water reservoirs. Residual explosives have the potential to move into soils as well as surface and ground water and affect various ecological and human receptors. Therefore, U.S. Environmental Protection Agency (USEPA) has included seven nitro-substituted explosives including TNT and RDX as priority pollutants. Labscale studies have revealed that TNT, RDX, and HMX are toxic to a wide spectrum of organisms including bacteria, algae, plants, earthworms, mammals, and humans. No doubt, traditional treatments of ammunition wastes, like open detonation and burning, adsorption onto activated carbon, photo-oxidation, etc., are not only costly, but also damaging the environment. Therefore, scientists are interested to develop an alternative technology based on microbes and plants which will be not only cost-effective, but also environment friendly.

In view of above facts, the editor has made his sincere efforts to compile the latest developments on biological remediation of explosive residues in an edited volume which will serve as a ready reckoner to the scientists, policy makers, teachers and students, and military personnel for the remedial measures to decontaminate the explosive residues in soils and waters by microbes and plants, alone or in combination.

In this endeavor, I would like to profusely thank all the contributors for their prompt response and active participation by contributing a review article on different aspects of biological degradation of explosive residues. I would also like to acknowledge my Ph.D. students associated with me Ms. Shweta Mishra, Mrs. Babita Kumari and Ms. Nitanshi Jauhri for their academic and technical support. Besides, untiring service, provided by Mr. Dilip Chakraborty in preparing the manuscript meticulously, is heartily acknowledged.

Lastly, I would also like to thank my family members: Mrs. Manorama Singh (wife), Ragini (daughter), and her kids Antra and Avantika and Pritish (son) for their inspiration, endurance, and moral support to me in this endeavor.

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Biodegradation of Nitrophenol Compounds

Nobutada Kimura, Wataru Kitagawa and Yoichi Kamagata

1 Introduction

Nitrophenol compounds have been used in a number of ways in medicines, explosives, and pesticides (Munnecke 1976). Due to the intense yellow color of the phenolate anion and pH reactivity [*p*-nitrophenol (PNP) is colorless at pH5.6 and the corresponding phenolate anion has a maximum yellow color at pH 7.6], nitrophenols are often used directly in titrations as indicators. Nitrophenols are also used in monitoring the enzyme activity, such as ß-galactosidase activity through detection of nitrophenol moiety and concomitant formation of the yellow color. Picric acid, also called 2,4,6-trinitrophenol, has been extensively used as a military explosive. In addition picric acid has been also used as a yellow dye, as an antiseptic and in the synthesis of chloropicrin, or nitrotrichloromethane, CCl_3NO_2, a powerful insecticide. A wide use of nitrophenol compounds and their subsequent release leads to environmental pollution. The US Environmental Protection Agency (USEPA) adds several mononitrophenols, dinitrophenols, and 2,4,6-trinitrophenol (picric acid) to its Emergency Planning and Community Right-to-Know Act (EPCRA) list of hazardous and toxic chemicals (EPA 1995). Due to this potential toxicity and persistence in the environment, to know the fate of the compounds and to establish the technologies for rapid remediation detoxification of these compounds are necessary. This chapter has focused on the microbial degradation of three isomers of nitrophenol.

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2 Biodegradation of Nitroaromatic Compounds by Microorganisms

Bacteria have evolved a variety of aerobic strategies for the removal of the nitro-group during conversion of the nitroaromatic compouds to central metabolites (Fig. 1).

Microbial degradation of nitrophenol compounds by microbial enzymes has been reported by several workers (Sudhakar et al. 1978; Hess et al. 1990; Dickel and Knackmuss 1991; Spain and Gibson 1991; Ecker et al. 1992; Groenewegen et al. 1992; Lenke et al. 1992; Nishino and Spain 1993; Rhys-Williams et al. 1993; Haigler et al. 1994; Jain et al. 1994; Nadeau and Spain 1995; Nishino and Spain 1995; Meulenberg et al. 1996; Rajan et al. 1996; Schafer et al. 1996, 1997, 1999; Michan et al. 1997; Kadiyala and Spain 1998; Spiess et al. 1998; Behrend and Heesche-Wagner 1999; Ebert et al. 1999; Katsivela et al. 1999; Rieger et al. 1999; Zhao and Ward 1999; Bhushan et al. 2000; Kimura et al. 2000; Shinozaki et al. 2002; Kuda et al. 2011; Kristanti et al. 2012).

Different enzymes involved in biodegradation of various nitrophenol compounds have been listed as below:

1. Monooxygenase: 2-Nitrophenol,4-Nitrophenol, 4-Nitroanisole
2. Dioxygenase: Nitrobenzene, 2-Nitrotoluene,3-Nitrobenzoate, 1,3-Dinitrobenzene, 2,6-Dinitrophenol
3. Reductase: 2,4-Dinitrophenol, 2,4,6-Trinitrophenol
4. Mutase: Nitrobenzene,3-Nitrophenol,2-Chloro-5-nitrophenol, 4-Chloronitrobenzene
5. Hydroxylaminolyase: 4-Nitrotoluene, 4-Nitrobenzoate, 3-Nitrophenol

The most widely studied approach involves the initial oxidative removal of the nitro group as nitrite in a reaction catalyzed by a monooxygenase enzyme. Some bacteria eliminate a nitro group following initial dioxygenation to a dihydroxy intermediate. Aerobic bacteria attack 2,4-dinitrophenol (24DNP) and

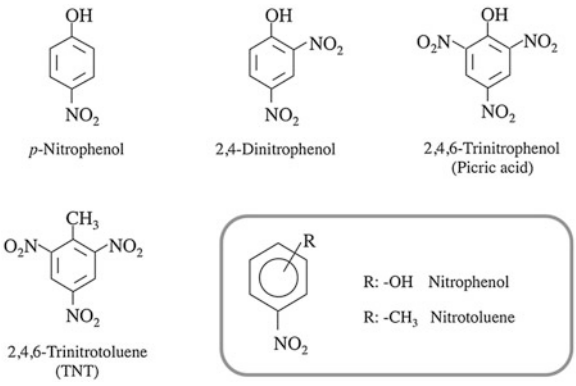

Fig. 1 Nitroaromatic compounds

2,4,6-trinitrophenol through formation of a hydride-Meisenheimer complex before elimination of the first nitro group as nitrite. A complete reduction of the nitro group to the amine does not appear to be a mechanism that is widely used by aerobic bacteria for productive metabolism. In one mechanism, the hydroxylamino compound is attacked by an enzyme hydroxylaminolyase, resulting in the production of the corresponding catechol and elimination of ammonia.

Bacteria, which were able to use nitrophenol compounds as a sole carbon or nitrogen source were isolated by enrichment technique from environmental samples. Although a few studies on the microbial ecology and molecular evolution were performed, they are ubiquitous at very low numbers in the contaminated soil (Hanne et al. 1993; Kimura et al. 2000).

2.1 Nitrophenol

2.1.1 *p*-Nitrophenol

PNP is very important compound as a basic material for medicines, dyes, and explosives. This compound is used on a large scale in the synthesis of the aspirin substitute acetaminophen and in the manufacture of pesticides, such as parathion and methylparathion (Spain and Gibson 1991; Zylstra et al. 2000). In the environment, such pesticides are hydrolyzed and transformed to 4-NP (Munnecke and Hsieh 1974, 1976; Sharmila et al. 1989). The toxicology of 4-NP has been studied and reviewed by the Agency for Toxic Substances and Disease Registry (1992). PNP irritates the eyes, skin, and respiratory tract leading to inflammation of these parts. PNP has a delayed interaction with blood and forms methaemoglobin, which is responsible for methemoglobinemia, potentially causing cyanosis, confusion, and unconsciousness.

Microbial degradation of PNP has been described by several bacteria including *Arthrobacter, Bacillus, Flavobacterium, Moraxella, and Pseudomonas* (Raymond and Alexander 1971; Nelson 1982; Heitkamp et al. 1990; Spain and Gibson 1991). At an early stage of the research, Simpson and Evans (1953) reported that *Pseudomonas* bacteria converted PNP into hydroquinone in association with the production of NO_2^- (Simpson and Evans 1953). Until now, several 4-NP-degrading bacteria have been isolated, and their degradation pathways have been also studied. As shown in Fig. 2, the two major initial degradation pathways of 4-NP have been characterized. The degradation pathway, in which 4-NP is converted to maleylacetate via hydroquinone (hydroquinone pathway) (Fig. 2, top), was preferentially found in gram-negative bacteria, such as *Burkholderia* spp. and *Moraxella* spp. (Spain and Gibson 1991; Prakash et al. 1996). The degradation pathway, in which 4-NP is converted via 4-nitrocathechol (4-NCA) and hydroxyquinol (hydroxyquinol pathway) (Fig. 2, bottom), was preferentially found in gram-positive bacteria, such as *Bacillus* spp. and *Arthrobacter* spp. (Jain et al. 1994; Kadiyala and Spain 1998). Besides, anaerobic degradation of PNP has been

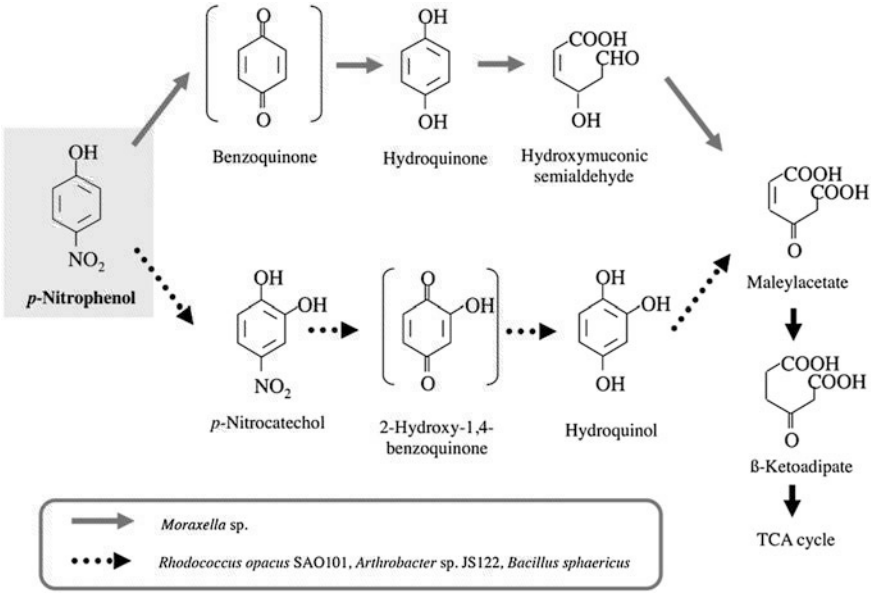

Fig. 2 Proposed pathway for PNP degradation by bacteria

reported. PNP was reductively converted into *p*-Aminophenol by *Desulfovibrio gigas* and *Clostridium pasteurianum* (Gorontzy et al. 1993). Complete mineralization of *p*-nitrophenol to CH_4 and CO_2 was observed after extended incubation period in the anaerobic sewage sludge diluted to 10 % in a mineral salts medium (Boyd et al. 1983).

Several studies of genetic information related to 4-NP degradation have been carried out. In association with the hydroquinone pathway, 4-NP degradation genes were cloned from *Pseudomonas* sp. strain ENV2030 and *Pseudomonas putida* JS444 (Zylstra et al. 2000). Similarly, in the case of hydroxyquinol pathway, 4-NP catabolic genes were cloned from *Rhodococcus opacus* strain SAO101, *Arthrobacter* sp. strain JS443, and *Rhodococcus* sp. strain PN1 (Kitagawa et al. 2004; Perry and Zylstra 2007; Takeo et al. 2008; Yamamoto et al. 2011). A gram-positive 4-NP degrader,*R. opacus* strain SAO101, was isolated from a sub-tropical island in Japan, which was able to utilize PNP as a sole carbon source (Kimura and Urushigawa 2001). As shown in Fig. 3, the degradation pathway of strain SAO101 followed (hydroxyquinol pathway) in which 4-NP is converted via 4-nitrocathechol (4-NCA) and hydroxyquinol. To obtain genetic information, a novel 4-NP degradation gene cluster from a gram-positive bacterium, *Rhodococcus opacus* SAO101, was identified and characterized (Kitagawa et al. 2004). The deduced amino acid sequences of *npcB, npcA,* and *npcC* showed similarity with phenol 2-hydroxylase component B (reductase, PheA2) of *Geobacillus thermoglucosidasius* A7 (32 %), 2,4,6-trichlorophenol monooxygenase (TcpA) of *Ralstonia eutropha* JMP134 (44 %), and hydroxyquinol 1,2-dioxygenase (ORF2) of *Arthrobacter* sp. strain BA-5-17 (76 %).

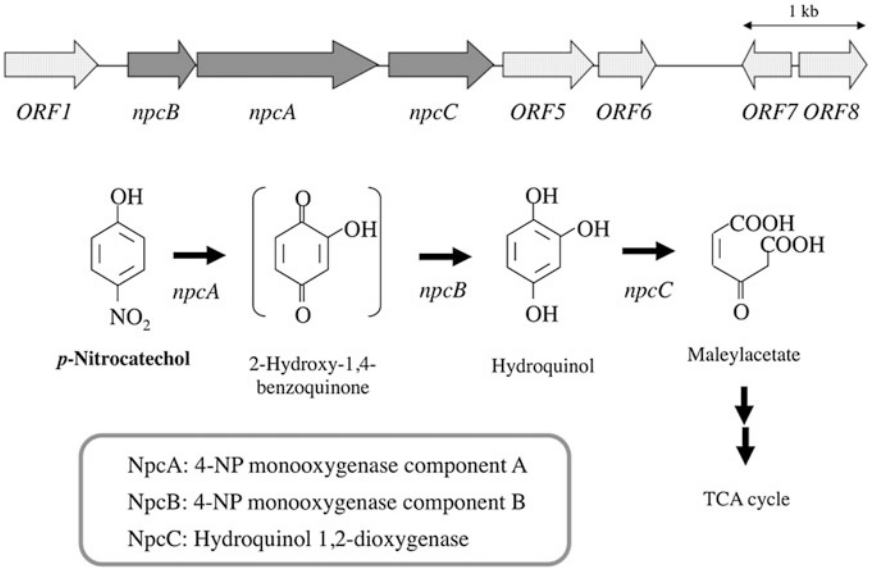

Fig. 3 Metabolic pathway for degradation of *p*-Nitrophenol by *Rhodococcus opacus* strain SAO101

This combination of degradative genes mixture converted 4-NP to hydroxyquinol and 4-nitrocatechol (4-NCA) to hydroxyquinol. Furthermore, the crude cell extract of *E. coli* containing pETnpcC converted hydroxyquinol to maleylacetate. These results suggest that *npcB* and *npcA* encode the two-component 4-NP/4-NCA monooxygenase while *npcC* encodes hydroxyquinol 1,2-dioxygenase.

Many aromatic degradation genes are known to be encoded on plasmid DNAs (Worsey et al. 1978; Don and Pemberton 1981; van der Meer et al. 1991; Prakash et al. 1996; Romine et al. 1999; Chauhan et al. 2000; Vedler et al. 2000). In particular, several *Rhodococcus* strains harbor aromatic degradation genes on large linear plasmids (Dabrock et al. 1994; Kosono et al. 1997; Shimizu et al. 2001). Three strains of 4-NP degrading bacteria, i.e., *Arthrobacter protophomiae* RJK100 (Chauhan et al. 2000), *B. cepacia* RJK200 (Prakash et al. 1996), and *Arthrobacter aurescens* TW17 (Hanne et al. 1993), have been reported to harbor plasmids involved in 4-NP degradation. The pulsed-field gel electrophoresis (PFGE) analysis revealed that strain SAO101 had three large linear plasmids, designated pWK301 (1,100 kb), pWK302 (1,000 kb), and pWK303 (700 kb) (Kitagawa et al. 2004; Kimura et al. 2006). By Southern hybridization analysis, a unique positive hybridization signal was observed at the position of origin of electrophoresis (data not shown). This result indicated that the *npc* genes were encoded on chromosomal DNA.

The authors also isolated PNP-degrading bacteria by aerobic batch enrichment from a laboratory-scale PNP-degradation reactor using a mineral salt medium containing a low and high PNP concentration (Shinozaki et al. 2002). They isolated two bacteria species, *Pseudomonas* sp. YTK17 and *Rhodococcus opacus* YTK32, that utilize PNP as their sole carbon source of carbon and energy. These strains exhibited differences in PNP degradation activity in relation to PNP concentration. Strain YTK17 showed a high level of degradation following pre-exposure to a low PNP concentration, whereas strain YTK32 required a relatively high PNP concentration for degradation to occur. These results indicated that phylogenetically and physiologically different types of PNP-degradation bacteria co-existed in a reactor.

2.1.2 2,4-Dinitrophenol

2,4-Dinitrophenol (2,4-DNP) is well known as an "uncoupler" compound (Hanstein 1976). 2,4-DNP can cross membranes in its protonated form, acting as a H^+ carrier and dissipate the electrochemical gradient across cell membranes, thus uncoupling the oxidative phosphorylation pathway without blocking oxygen consumption (Alberts et al. 1989). The ability of 2,4-DNP to function as a respiratory uncoupler causes toxicity to microorganisms (Bruhn et al. 1987), and recommends restricting its concentration in the natural waters (Keith and Telliard 1979).

2,4-DNP-degrading strains, which have ability to utilize 2,4-DNP as a sole carbon source, mainly belong to high GC gram-positive bacteria with one exception, *Janthino bacterium* which used 2,4-DNP as a nitrogen source (Lenke et al. 1992; Rhys-Williams et al. 1993; Blasco et al. 1999). Based on the systematic characterization of microorganisms that degrade 2,4-DNP, the authors proposed that the isolated 2,4-DNP-degrading strains could be classified as two different kinds of bacteria, *Rhodococcus, Nocardia,* and *Nocardioides* strains based on phylogenetic and phenotypic properties (Kimura et al. 2000).

Generally, microbial degradation in reactors has been used as a tool for the treatment of 2,4-DNP contaminated wastewater (Hess et al. 1990; Xing et al. 1995, 1999; Gisi et al. 1997). However, it takes a long time for microorganisms to degrade 2,4-DNP in reactors due to the toxicity of the compound (Takahara 1980). Knowledge about the microbial community in the 2,4-DNP digesting reactor is useful for establishing the operational conditions needed to degrade this compound efficiently. The microbial community of a 2,4-dinitrophenol digesting reactor was investigated using molecular biological techniques based on 16S rDNA gene sequences. A PCR-denaturing gradient gel electrophoresis (DGGE) analysis of the bacterial community in the reactor showed that species of *Rhodococcus, Nocardioides,* and *Nitrospira* species were dominant in the reactor. The isolation and phylogenetic analysis of 2,4-DNP-degrading bacteria from the reactor revealed that isolated bacterial strains were found of two types having different 16S rDNA sequences. One type of these strains was identified as relative of *Rhodococcus*

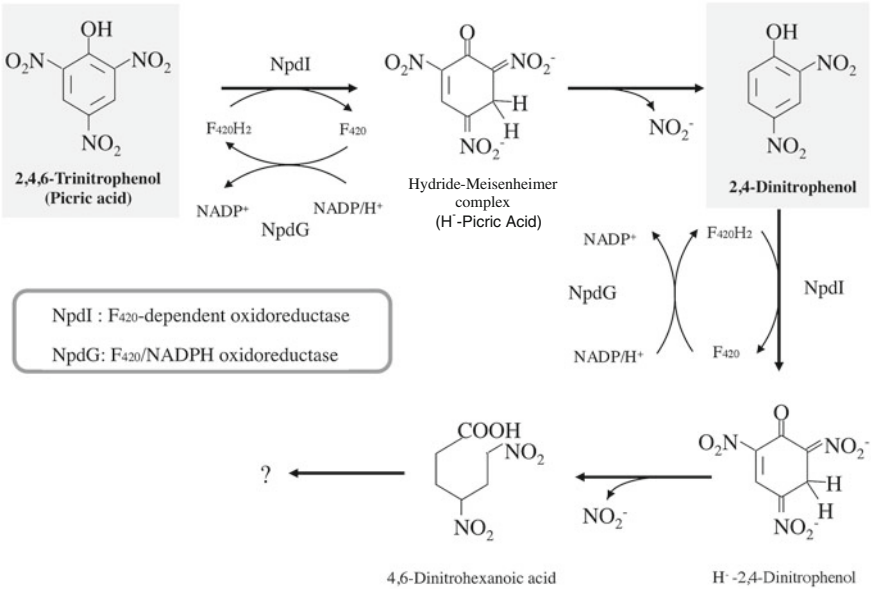

Fig. 4 Proposed degradation pathway for picric acid and 2,4-Dinitrophenol by *Rhodococcus* strains

koreensis, while an other type was a relative of *Nocardioides simplex* FJ21-A (Kimura et al. 2003).

Although degradation of mononitroaromatic compounds and some dinitroaromatic compounds, such as 2,6-DNP, is initiated by oxygenation by a monooxygenase or dioxygenase (Zeyer JaK 1984; Spain and Gibson 1991; Ecker et al. 1992; Jain et al. 1994; Meulenberg et al. 1996; Schenzle et al. 1997), the analysis of metabolization of trinitroaromatic compounds revealed that trinitroaromatic compounds, such as 2,4,6-trinitrotoluene (TNT) and 2,4,6-trinitrophenol (picric acid), are reduced at an initial step of bacterial degradation, which is readily susceptible to an initiation of reductive rather than oxidative attack in the presence of electron withdrawing nitro groups as substituents (Fig. 4) (Lenke et al. 1992; Vorbeck et al. 1998; Behrend and Heesche-Wagner 1999; Rieger et al. 1999). In 2,4-DNP degradation strains, the reductive pathway which converted 2,4-DNP with concomitant liberation of stoichiometric amounts of nitrate and 4,6-dinitrohexanoatewas as found in *Rhodococcus erythropolis* HL24-1 and HL24-2 (Lenke et al. 1992).

2.1.3 2,4,6-Trinitrophenol (Picric Acid)

2,4,6-Trinitrophenol (TNP) is used to be a main source of the military explosives. Picramic acid (2-Amino-4, 6-dinitrophenol), which is produced by the reduction of

picric acid, is used in the manufacturing of azo dyes. Historical use of this explosive by military and industries results soil and groundwater contamination. The Department of Defense (DoD) estimates that more than thousand of sites are contaminated with explosives and TNP (Walsh 1993; Thorne 1995). The EPA's Toxic Release Inventory (TRI) indicates that approximately 7.4 million pounds of TNP was released to the environment in the United States from 1988 to 2002 during industrial activities (TRI 2002).

TNP-degrading strains, which have the ability to utilize TNP as a sole carbon source, generally belong to high GC gram-positive bacteria (Lenke and Knackmuss 1992; Behrend and Heesche-Wagner 1999; Ebert et al. 1999; Walters et al. 2001). The initial degradation step of TNP is very unique. $F_{420}H_2$-dependent reductive attack on the aromatic ring creates H^--Picric acid (the Hydride-Meisenheimer complex) of picric acid which is further converted into 2,4-DNP through the release of nitro group catalyzed by the F_{420}-dependent reductase (NpdI) enzyme (Fig. 4). Hydride-Meisenheimer complex of 2,4-dinitrophenol (H^--2,4-DNP) was identified as an intermediate of picric acid degradation by *Nocardioides* sp. strain CB 22-2 (Alberts et al. 1989; Ebert et al. 2001). This is feasible by assuming that nitrite is released either from H^--2,4-DNP or a tautomer of the protonated H^--2,4-DNP. Following nitrite release and hydrolytic ring cleavage, two metabolites were proposed 4,6-dinitrohexanoate (Lenke and Knackmuss 1996) and 3-nitroadipate

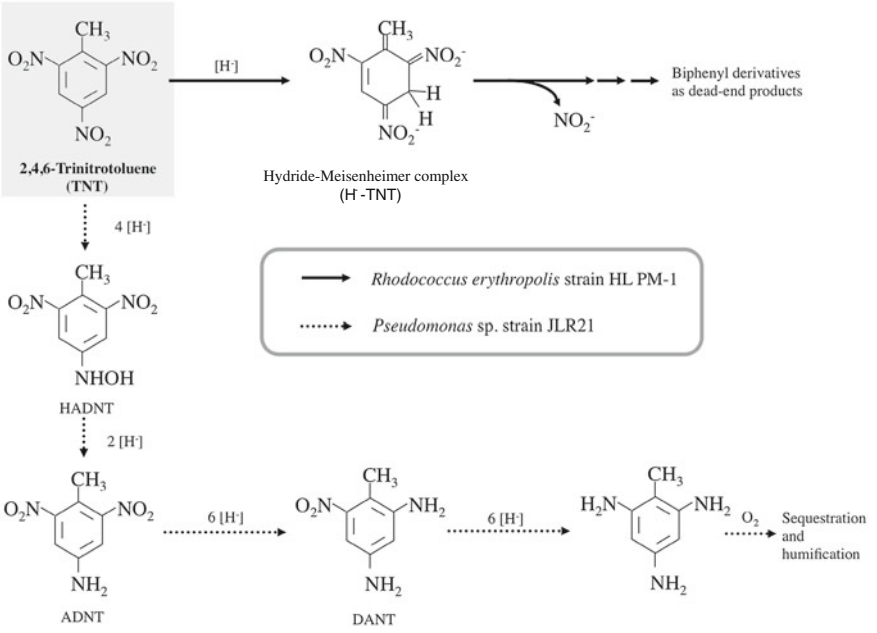

Fig. 5 Proposed degradation pathway for Trinitrotoluene (TNT) by *Rhodococcus erythropolis* strain HL PM-1 and *Pseudomonas* sp. strainJLR21. *HADNT* Hydroxylamino dinitrotoluene, *ADNT* Amino dinitrotoluene, *DANT* Diamino dinitrotoluene

(Blasco et al. 1999). Several of the *npd* genes of *R. (opacus) erythropolis* HL PM-1, showing sequence similarities to enzymes responsible for the β-oxidation of fatty acids (Russ et al. 2000), might be involved in the oxidation of 4,6-dinitrohexanoate. TNP degradation capacity of *R. (opacus) erythropolis* HL PM-1 is inducible by 2,4-DNP (Russ et al. 2000; Walters et al. 2001) but not by picric acid. TNP-degrading strain *R. erythropolis* D3213 was shown to have a TNP degradation pathway. This strain D3213 degrade TNP and 2,4-DNP constitutively. These results suggested that strain HL PM-1 and D3213 had different abilities to degrade TNP.

Strain HL PM-1 possessed reductive enzyme systems, which catalyze ring hydrogenation, i.e., the addition of a hydride ion to the aromatic ring of 2,4,6-Trinitrotoluene (TNT) (Fig. 5). The hydride-Meisenheimer complex thus formed (H^--TNT) was further converted to a yellow metabolite, which by electrospray mass and nuclear magnetic resonance spectral analyses, was established as the protonated dihydride- Meisenheimer complex of TNT ($2H^-$-TNT) (Vorbeck et al. 1998).

3 Conclusion

In this chapter, current knowledge on the microbial degradation of nitrophenols was reviewed focusing on the genetic and biochemical information of nitrophenol degrading bacteria. The knowledge of microbial degradation of nitrophenols may serve as a basis for the use of bioremediation systems for the removal of nitrophenol. The future direction of research in this area of nitropehnol degradation is to make the appropriate bioremediation strategy for a nitrophenol-contaminated site. To make strategy for bioremediation of nitrophenols, the fate of the targeted pollutant in the environment must be well understood. Second, information on the toxicity of intermediates produced during the microbial degradation of nitrophenols is crucial for selecting the appropriate degradation pathway to be stimulated at the remediation site. To understand the potential fate of intermediates of nitrophenol degradation, the complete pathway for nitrophenol degradation needs to be elucidated and the metabolites of nitrophenol degradation have to be identified. Third, information on the biodegradability of nitrophenols by natural microbes in the environments must be obtained. In the literature, knowledge on the natural consorcia for microbial degradation of nitrophenols in the environment has been not presented.

In addition, only a few reports of gene sequence are known from selected catabolic pathways and microorganisms. The sequence information in the database needs to be increased to include genes for all of the possible pathways for nitrophenol degradation, by different microorganisms. The information of the different gene sequences for nitrophenol degradation is useful for understanding the evolution of pathways to solve question how two different pathways were evolved in PNP-degrading strains. Furthermore, it is an important question to be answered whether bacteria, that degrade nitropheols, have evolved mechanisms for

protection against these compounds. PNP and TNP are known to be toxic to microorganisms. Knowledge of the protection mechanism for nitrophenols can be used for the remediation of the environment so that the microorganisms can degrade the nitrophenol compounds efficiently and effectively.

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Microbial Degradation of 2,4,6-Trinitrotoluene *In Vitro* and in Natural Environments

Harald Claus

1 Introduction

2,4,6-Trinitrotoluene (TNT) is a nitroaromatic explosive that was released into soil and water ecosystems mainly due to its massive use during the two world wars. As a result, many sites used for TNT production have become seriously contaminated with the explosive and related compounds (Fuller et al. 2004; Lewis et al. 2004). Typical contaminated sites may contain up to 10 g/kg TNT in soils and up to 100 mg/l in water. TNT and its metabolites exhibit a high toxic and mutagenic potential on both prokaryotes and eukaryotes (Spanggord et al. 1995; Honeycutt et al. 1996; Lachance et al. 1999; Maeda et al. 2006). Consequently, there is an urgent need to clean up contaminated sites to ensure environmental quality and safety. It has been estimated that nearly 3,200 sites in Germany require environmental restoration (Preuß and Eitelberg 1999). Various physical/chemical procedures for remediation of TNT contaminated soils have been established, but all are very cost-intensive. Carbon adsorption has often been used for removal of nitroaromatics from contaminated ground- and surface-waters (Wujcik et al. 1992). Unfortunately, the matrix is expensive and the spent carbon still constitutes a problematic waste (Schmidt et al. 1998).

Biological based remediation is an economical and ecological compatible approach to detoxify areas contaminated with xenobiotics (Alvarez and Illman 2005; Crawford et al. 2005; Singh and Tripathi 2006). However, TNT is resistant to oxidative microbial degradation and only low mineralization rates have been sporadically reported with bacterial consortia and several white-rot fungi. The reason is the presence of three electron withdrawing nitro-groups in TNT which introduce steric constraints and confer a high electron deficiency to the aromatic ring (Heiss and Knackmus 2002). Instead of oxidation, many bacteria catalyse the

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reduction of one or two nitro-groups of TNT to monoaminodinitrotoluenes (ADNT) and diaminonitrotoluenes (DANT). Another pathway is mediated by addition of one or two hydride ions to the aromatic ring, resulting in the formation of Meisenheimer-complexes (adducts of aromatic nitro-compounds with a nucleophile) often accompanied by release of nitrite. The electron transfer is catalyzed by different types of cytoplasmatic nitroreductases (Pak et al. 2000; Kim and Song 2005). Reactive nitroso- and hydroxylamino-intermediates can further react to condensated azoxy-dimers and acetyl-derivates of TNT. Under strictly anaerobic conditions, ADNT is further reduced to 2,4,6-triaminotoluene (TAT) which is highly reactive and can polymerize or irreversibly bind to the organic soil matrix (Thiele et al. 2002).

The reductive reactions are the basis of several treatment processes for the bioremediation of TNT-contaminated soils (Breitung 1996; Lenke et al. 1998, 2000; Fuller et al. 2004; Lewis et al. 2004). However, there is a lack of biological strategies to clean up contaminated water ecosystems. Some promising microbiological approaches for detoxification of aquatic environments are addressed in this chapter.

2 2,4,6-Trinitrotoluene

2.1 Toxicity

Due to its high blasting power and relative security of handling, TNT is still one of the most used military explosives. It has been estimated that around 1.000,000 kg TNT is produced per year (Singh et al. 2012). Five hundred thousand US gallons of water, contaminated with TNT and other nitroaromatic compounds, may be released into the environment by one TNT-manufacturing plant in one day. In USA, 15 million acres at over 2000 sites are suspected or known to be contaminated with military munitions (Montgomery et al. 2011). At some munitions manufacturing and processing sites, the contamination can be as high as 200 g TNT per 1 kg of soil (Symons and Bruce 2006).

TNT forms yellow crystals and has a water solubility of 130 mg/l. At contaminated sites, it exists as a fine dust, flakes or crystallized chunks. Its heterogeneous distribution in soil restricts mobility, microbial degradation and also its analysis.

In several studies, it has been demonstrated that TNT and most of its degradation products are toxic to fish (Osmon and Klausmeier 1972), rats and mice (Ashby et al. 1985) as well as to algae and aquatic plants (Sunahara et al. 1999). For microorganisms, such as yeasts, actinomycetes and Gram-positive bacteria, TNT is toxic at concentrations of ca. 50 mg/l (Klausmeier et al. 1973). Also precursors and metabolites of TNT are classified as very toxic, carcinogenic and mutagenic (Schneider et al. 2000; Haarck et al. 2001). Ribeiro et al. (2012)

reported that the toxic potential of effluents generated by TNT production (yellow and red waters), produced from a plant located in Brazil was extremely high to all test organisms (*Daphnia similis, Danio rerio, Escherichia coli, Pseudomonas putida* and *Pseudokircheneriella subcaptata*).

Numerous symptoms of poisoning in humans following inhalation or dermal absorption of mononitrotoluene, dinitrotoluene, and TNT are observed a few days after exposure: headache, loss of appetite, dizziness, nausea, insomnia, numbness of various parts of the skin and diarrhea. Strong changes in the hemogram are the result of exposure. A particularly striking symptom is cyanosis, a bluish-red discoloration of lips, fingernails and skin due to oxygen deficiency. That is caused by reduced metabolites of TNT which are blamed for increased methemoglobin formation and hemolysis. The metabolites of TNT cause liver damaging effects (Koss et al. 1989).

2.2 Microbial Degradation of TNT

The degradation of TNT by microorganisms has been extensively studied for many years and the results have been compiled in numerous reviews (Fritsche et al. 2000; Hawari et al. 2000; Lenke et al. 2000; Spain et al. 2000; Esteve-Núñez et al. 2001; Rodgers and Bunce 2001; Rosser et al. 2001; van Aken and Agathos 2001; Heiss and Knackmus 2002; Fuller et al. 2004; Lewis et al. 2004; Schrader and Hess 2004; Zhao et al. 2004; Stenuit and Agathos 2010; Rylott et al. 2011). Some basic reactions are listed in Table 1.

2.2.1 TNT Degradation by Bacteria

There is one major problem with microbial TNT degradation: the three symmetrically arranged nitro-groups induce a high electron deficiency at the aromatic ring. An oxidative degradation and the use of TNT as a source of carbon and energy is extremely unlikely. Thus, the term degradation in this context means transformation or destruction of TNT, but not mineralization, i.e., use as the sole growth substrate for a microorganism.

The initial metabolites in the biotransformation of TNT are hydroxylaminodinitrotoluenes (HADNTs) aminodinitrotoluenes (ADNTs), diaminomononitrotoluenes (DANTs) and tetranitroazoxytoluenes (AZTs) (Hawari et al. 2000; Oh et al. 2000). Because of the electron deficiency of the ring π system, the initial degradation of TNT by microorganisms is characterized by reductive reactions (Vorbeck et al. 1998). The nitro-moieties of TNT ($-NO_2$) can be successively reduced to nitroso ($-NO$), hydroxylamino ($-NHOH$) and finally amino ($-NH_2$) groups (Fig. 1). By strictly anaerobic bacteria, such as *Clostridium* sp., *Desulfovibrio* sp. and archaebacteria as *Methanococcus* sp., TNT is completely reduced

Table 1 Degradation of TNT by microorganisms

Mechanism	Products*
Primary enzymatic reactions	
Stepwise reduction of nitro-groups of the aromatic ring by nitroreductases	Nitrosodinitrotoluene Hydroxyldinitrotoluene Aminodinitrotoluene Diaminodinitrotoluene
Hydride addition to the aromatic ring by flavoproteins of the *old yellow enzyme* family (*OYE*)	Monohydride– Dihydride- Protonated Dihydride- Meisenheimer complex
Secondary abiotic reactions	
Condensation of NODNT/ HADNT	Tetranitroazoxytoluene Secondary Diarylamines
Condensation of HADNT/$2H^-$ TNT.H^+	
Covalent binding to cell compounds	Protein adducts
Secondary enzymatic reactions	
Oxidation of the methyl-group	
Acetylation of an amino-group	
Oxidation of reduced metabolites (ADNT, DANT) by fungal exoenzymes and coupling to organic soil components	Polymers Humic acids adducts

*Different isoforms are formed depending on the microorganism and enzyme specificity

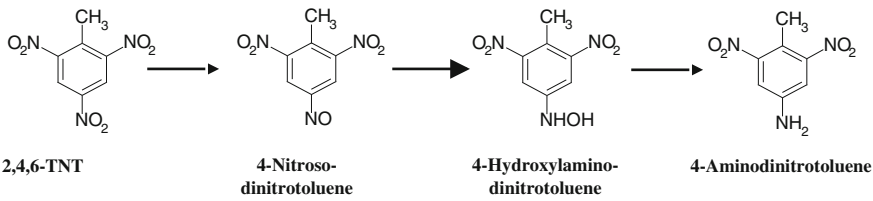

Fig. 1 Microbial transformation of TNT by reduction of nitro-groups

to 2,4,6-triaminotoluene (Boopathy and Kulpa 1994; Regan and Crawford 1994; Crawford 1995; Ederer et al. 1997).

These reduced TNT compounds present the primary products of the microbial metabolism. However, depending upon the reaction conditions (e.g., pH), they can be further converted by biotic and abiotic mechanisms to azo-, azoxy-, hydrazone-, and phenol-acetyl derivatives (Hawari et al. 2000). The intermolecular condensation of partially reduced derivatives leads to formation of azoxytetranitrotoluenes (Haidour and Ramos 1996).

The second important pathway is the direct reduction of the aromatic ring by addition of hydride-ions with the formation of monohydride-, and dihydride-Meisenheimer complexes (Fig. 2). As both pathways (nitro-reduction and aromatic

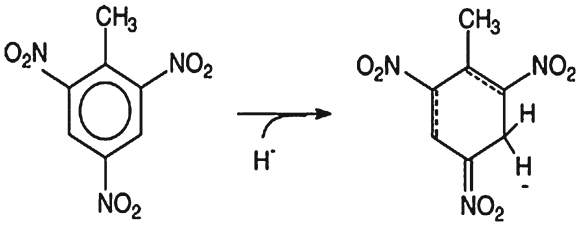

Fig. 2 Microbial transformation of TNT by hydride addition to the aromatic ring

2,4,6-TNT **Monohydride-Meisenheimer complex**

ring-reduction) may co-exist in the same cell, condensation reactions between dihydride-Meisenheimer complexes and hydroxylaminodinitrotoluene can lead to the formation of diarylamines with concomitant liberation of nitrite (Wittich et al. 2009).

TNT enters the bacterial cell most probably by passive diffusion across the cell barriers. In contrast, multi-drug efflux pumps are induced in *Pseudomonas putida* KT2440 in the presence of TNT, suggesting the importance of active extrusion systems in maintaining low intracellular TNT concentration to overcome toxicity (as reviewed by Stenuit and Agathos 2010). As a result of TNT degradation, different amounts of monomeric transformation products (ADNT, DANT) have been found extracellular (Claus et al. 2007a, b). Conclusively, active efflux systems may also exist for these compounds.

2.2.2 TNT Degradation by Fungi

The enzymatic conversions mentioned above do not imply ring opening (Hawari et al. 2000). This is the reason why TNT is transformed by bacteria, but usually not mineralized. However, white-rot fungi and the litter degrading fungus *Phanerochaete chrysosporium* as well as *Stropharia* species have been shown to mineralize TNT, at least in part, under aerobic conditions (Bumpus and Tatarko 1994; Fritsche 1998; Esteve-Núñez et al. 2001).

In a screening program, 91 fungal strains belonging to 32 genera of different ecological and taxonomic groups (wood and litter decaying basidiomycetes, saprophytic micromycetes) were tested for their ability to metabolize and mineralize TNT (Scheibner et al. 1997b). All these strains metabolized TNT rapidly by forming monoaminodinitrotoluenes (ADNT). Micromycetes produced higher amounts of ADNT than did wood and litter decaying basidiomycetes. A significant mineralization of (C^{14}) TNT was only observed for certain wood and litter decaying basidiomycetes. The most active strains, *Clitocybula dusenii* TMb12 and *Stropharia rugosa-annulata* DSM11372 mineralized 42 and 36 %, respectively of the initial added (C^{14}) TNT to (C^{14}) CO_2 within 64 days. However, micromycetes (deuteromycetes, ascomycetes, zygomycetes) were unable to mineralize (C^{14}) TNT significantly.