Chapter 2

Bioremediation of Nitroaromatic Compounds

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Additional information is available at the end of the chapter

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Abstract

Nitroaromatics are major pollutants released in the environment during the post-industrialization era and pose toxic effects to living organisms. Several bacterial strains have been isolated for the degradation of these nitroaromatic pollutants. Some of them have been used in field trial experiments for the removal of nitroaromatics from industrial water and groundwater. Very few bacterial pathways have been characterized at genetic and molecular levels. In this review, we cover all reported degradation pathways and their gene evolution. These studies for nitroaromatics clearly indicate that most of the involved genes have evolved from preexisting enzymes by using all means of gene evolution like horizontal gene transfer, mutation, and promiscuity principle. This information has been exploited for the creation of hybrid pathways and better biocatalysts for degradation.

Keywords: Nitroaromatics, biodegradation, gene evolution, oxygenase

1. Introduction

Microbes are empowered to degrade environmental pollutants under different conditions to perform unusual metabolic and physiological activities [1]. The metabolic versatility of the microbes helps them utilize a range of organic/inorganic compounds for their growth. This metabolic versatility has been exploited for human benefit in various industrial products like cheese (dairy industry), insulin, antibiotics (pharmaceutical industry), etc. Microbial enzyme systems have also been used for the development of suitable biocatalysts for green chemistry applications [2]. Whole microbes have also been tested for their potential in bioremediation [3]. Organic aromatic compounds have been the main source of pollution in several water bodies [4]. Thus, a remediation and degradation study of aromatics has been a focus of intensive study. In this review, we will focus only on nitroaromatic compounds.

1.1. Nitroaromatics

Nitroaromatic compounds have at least one nitro group attached to the aromatic ring, like nitrobenzene, nitrotoluenes, nitrophenols, etc. In nature, nitroaromatic compounds are mostly found in natural products from different plants, fungi, and bacteria [5, 6]. The best known example of this is chloramphenicol, which is produced by *Streptomyces venezuelae* [7–9]. The role of some nitroaromatic compounds in cellular signaling has also been established. For example, 2-nitrophenol and 4-methyl-2-nitrophenol are well-known pheromones for ticks that enable them to aggregate and attach to mammals [5, 10] (Figure 1).

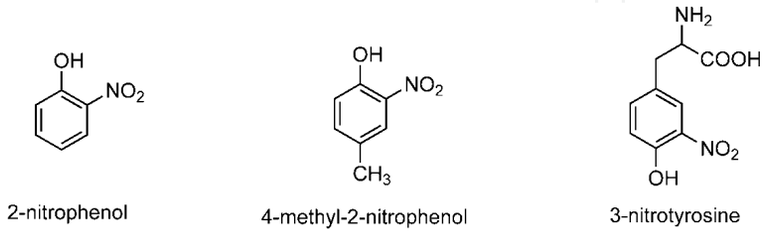

Figure 1. Nitroaromatics as cell signaling molecules.

In nature, several nitroaromatic compounds are produced by the incomplete combustion of fossil fuel, which releases hydrocarbons. These hydrocarbons produce nitroaromatic compounds after nitration with nitrogen dioxide present in the atmosphere. Mixtures of nitropolyaromatic hydrocarbons can be produced to form 3-nitrobiphenyl,1- and 2-nitronaphthalene,3-NT and nitrobenzene by a hydroxyl radical-initiated mechanism [11–15].

1.2. Synthetic nitroaromatic compounds (production and uses)

The versatile chemistry of the nitro group ensures that nitroaromatic compounds serve as important feed stocks in different industrial processes. These compounds are commonly used in the manufacture of pharmaceuticals. For example, substituted nitrobenzenes and nitropyridines are used in the production of indoles, which are active components of several drugs and agrochemicals [16]. Paracetamol (an analgesic and antipyretic) is synthesized in a one-step reductive acetamidation from 4-nitrophenol [17]. Nitrobenzenes or halonitrobenzenes are used in the synthesis of derivatives of phenothiazines, a large class of drugs with antipsychotic properties [18, 19].

Some nitroaromatics like nitrobenzene, nitrotoluene, and nitrophenols are used in the synthesis of pesticides. For example, fluorodifen [20], bifenox, parathion [21], and carbofuran [22] are synthesized from nitrophenols. Some dinitrophenols like 2,5-dinitro-*o*-cresol have been used in the synthesis of herbicides, insecticides, fungicides, etc. [5, 23].

Aromatic amines are the largest feedstock group for chemical industries. It is estimated that the worldwide consumption of aniline is approximately 3 million tons [5]. This consumption grew by 7% annually till 2014 and is expected to reach 6.2 million tons in 2015 (Global Analysts report 2014 on aniline production). Aniline is used in the synthesis of drugs, pesticides, and

explosives and used as a building block for the production of polyurethane foams, rubber, azo dyes, photographic chemicals, and varnishes [24].

Some nitroaromatics are used in the production of explosives like trinitrotoluene (TNT), which is produced by sequential nitration of toluenes. 1,3,5-Trinitrophenol (picric acid), which was prepared in 1771 as a yellow dye for fabrics [25], has also been used in explosive shells. The methyl group of TNT can be eliminated to produce trinitrobenzene (TNB), a high-energy explosive with decreased shock sensitivity [26].

1.3. Release of nitroaromatics in the environment

The estimated annual production of nitroaromatic compounds is 10^8 tons (http:www.ucl/agro/abi/gebi). Chemical industries release these compounds into the environment through various sources, like the use of pesticides and the improper handling or storage of chemicals. The leakage into industrial effluents by improper disposal or through accidental spills by explosive ammunitions are commonly responsible for these compounds to find their way into the environment [27]. A recent example of an industrial accident is from China, where about 100 tons of benzene and nitrobenzene were released to Songhua River because of an accidental explosion in the factory of China National Petroleum, Jilin City, on November 13, 2005 [28].

1.4. Toxic effects

The electron withdrawing property of the nitro groups creates a charge on the molecule. It is a unique property that makes the nitro group an important functional group for different industrial synthetic processes. Simultaneously, the same property makes these molecules hazardous to the environment. This is why these compounds are given hazardous rating 3 (HR 3), where 3 shows the highest level of toxicity [29]. These are toxic to most living organisms, including humans, fishes, algae, and microorganisms [30, 31]. Their toxicity principally manifests itself due to their ability to uncouple photo or oxidative phosphorylation processes [32, 33]. Some of these compounds are also known for their ecotoxicity [34, 35], immunotoxicity [36], carcinogenicity [37], mutagenicity [38, 39], and teratogenicity [40, 41]. Some nitroaromatics are also converted into carcinogens and mutagens when metabolized by liver or intestinal microflora [42, 43].

1.5. Treatment options

1.5.1. Physical, chemical, and physicochemical methods

Different physical methods are available for the treatment of these toxic chemicals, like adsorption, incineration, photo-oxidation, hydrolysis, volatilization, etc. During adsorption, these compounds are only adsorbed on resins and separated but not destroyed completely. In incinerations, these compounds are treated at very high temperatures, which is neither cost effective nor eco-friendly because toxic NO_x fumes are often released in the environment in this process. There are various reports on advanced oxidation processes (AOPs) that utilize ozone, UV radiation, hydrogen peroxide, or combinations of all these for the treatment of

nitroaromatic compounds [44–47]. The use of hydrogen peroxide in these processes generates toxic intermediates and is therefore not cost-effective [48]. Because of limitations of these methods, biodegradation has emerged as a viable alternative.

1.5.2. Bioremediation

Bioremediation involves biological agents to catalyze the degradation and transformation of recalcitrant molecules to simpler structures. Few common terms used in these processes are defined as follows:

- Biodegradation is the breakdown of organic pollutants due to microbial activity. In this process, the microbe feeds on the pollutant to grow. The degradation of contaminant generates energy and microbe utilizes this energy for its growth.
- Biomineralization is the process of complete biodegradation. The organic contaminants are degraded completely through a series of degradation steps and finally converted to inorganic molecules like H_2O and CO_2. In the process, organic molecule provides both carbon and energy to the microbe, and if organic molecule is nitroaromatic, it provides nitrogen as well.
- Biotransformation is the process where in one organic molecule is modified by the action of biological agents. Sometimes, biotransformation occurs with cometabolism, where a microbe uses a substrate for its growth but transforms another substrate, which is not utilized by microbe for its growth.

Microbes have been isolated from almost all the parts of biosphere. Further, their adoptability for different environmental conditions and ability to utilize even recalcitrant compounds for their foods make them suitable agents for bioremediation.

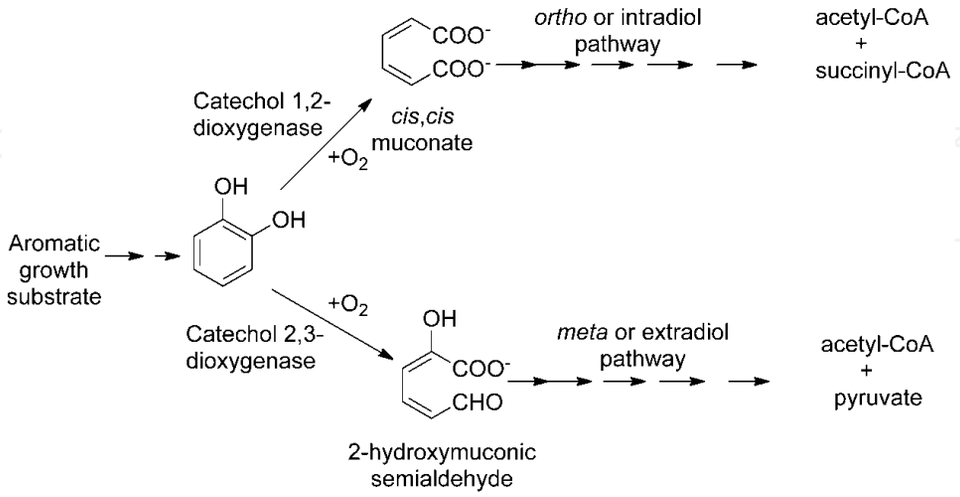

Figure 2. General principle of aerobic aromatic catabolism in bacteria. The three stages are as follows: the conversion of the growth substrate to catechol (or substituted catechol), then ring cleavage, and finally metabolism of the ring cleavage product to central metabolites by either the *ortho* or *meta* pathways. (Adapted from Williams and Sayers [51])

Biodegradation gained worldwide attention to treat toxic compounds [49]. This is because of its eco-friendliness. Supplementing the medium with readily utilizable carbon sources can enhance degradation processes. Thus, toxic intermediates are not generated and complete removal of toxic compounds is possible. During the last few decades, extensive research has been carried out for isolation of microbes with the abilities of degrading wide range of toxic nitroaromatic compounds and has been reviewed nicely [5, 49, 50]. Some common routes adopted by bacterial strains in nature during the degradation of aromatic compounds are described here.

1.6. General principles of pathways for aerobic aromatic catabolism

The pathway for catabolism of aromatic compound basically has three stages (Figure 2) [51–53]. In the first stage, the substrate undergo changes in its substituent groups by the action of mono- or dioxygenases to form catechols (or substituted catechols). The catechols then serve as substrates for the second stage of catabolism, that is, the ring opening. This process is facilitated by the action of dioxygenases, which breaks carbon–carbon bond by adding molecular oxygen and produce unsaturated aliphatic acid.

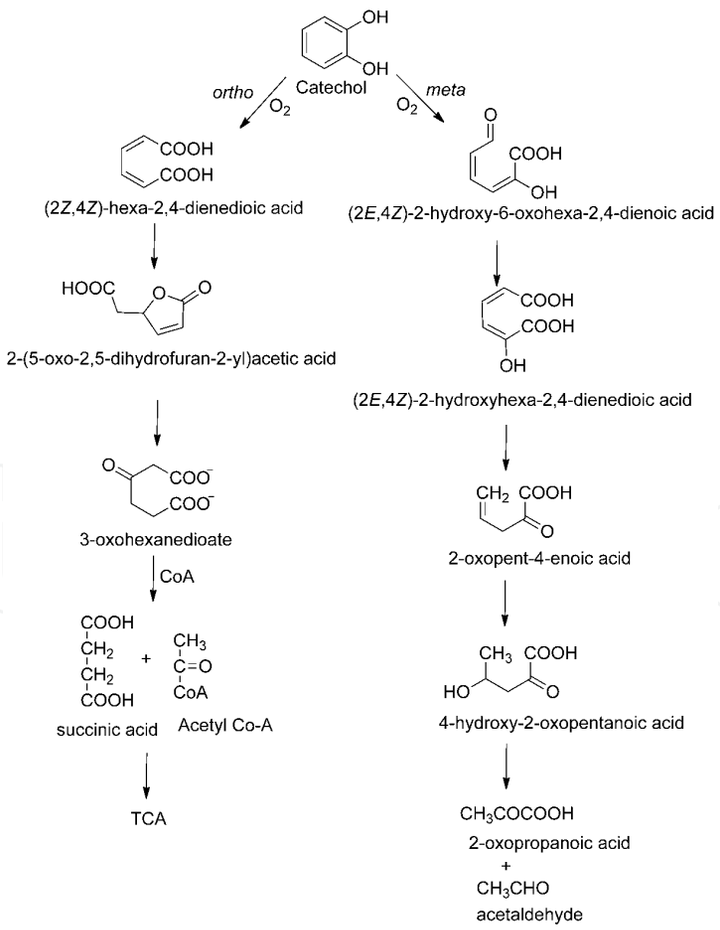

Figure 3. Central catechol pathway for the aerobic degradation of aromatics.

There are two families of ring opening enzymes, *ortho* or intradioldioxygenases, which produce *cis,cis* muconic acid (or its derivative), and *meta* or extradioldioxygenases, which produce 2-hydroxymuconic semialdehyde (or derivative). Both pathways (*ortho* and *meta* cleavage) are shown in Figure 3 and *meta* cleavage pathway for methylcatechols is shown in Figure 4. The third stage of catabolism is the conversion of the ring cleavage products into smaller compounds that can enter into central metabolic routes.

In general, aromatic compounds are initially catabolized by various pathways (known as peripheral pathways), which converge on a limited number of common intermediates (catechols or its derivatives). These intermediates are further utilized by a small number of common pathways (central pathways).

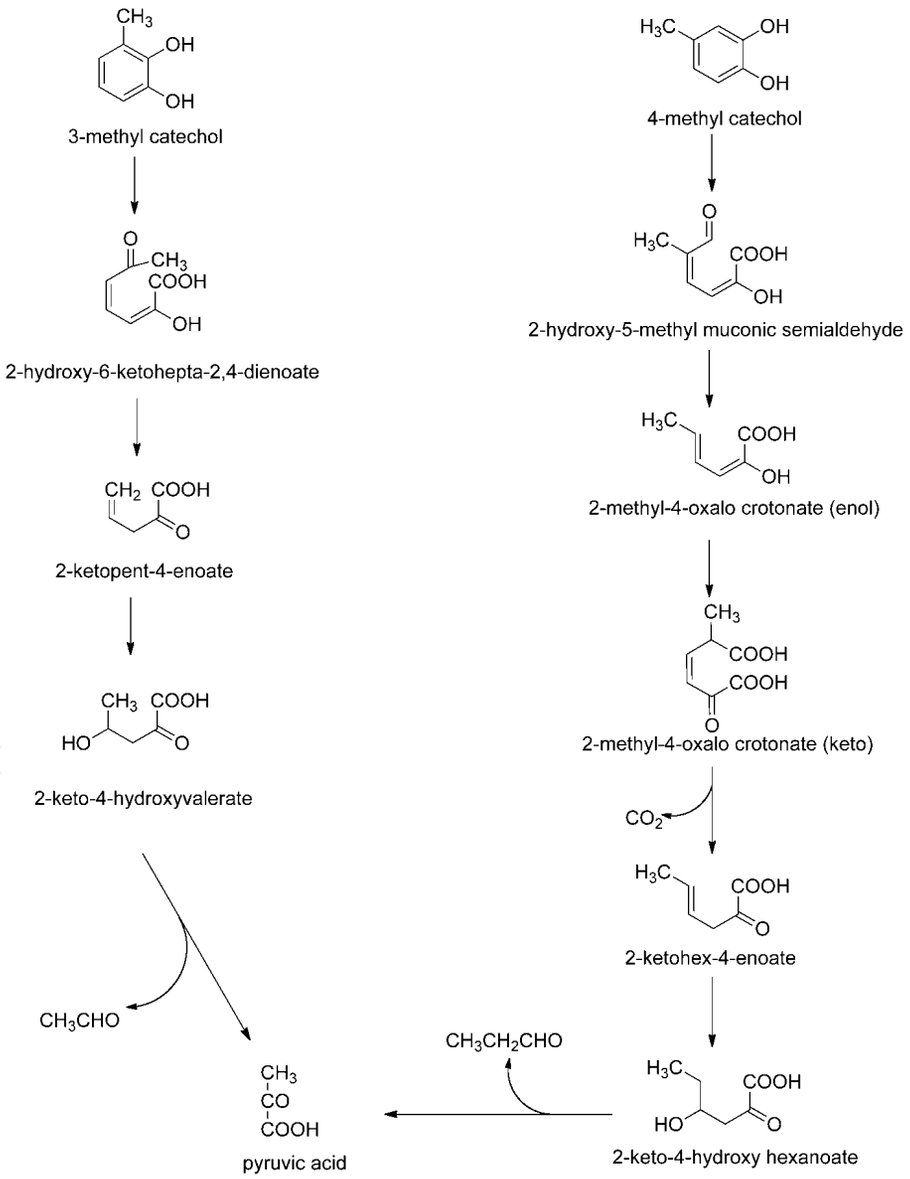

Figure 4. The *meta* cleavage pathway of methylcatechols.

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2. Microbial degradation of nitroaromatics

When a nitroaromatic compound is exposed to the environment, its biodegradation takes place either by anaerobic route or by aerobic route. Different strategies applied for the degradation of nitroaromatic compounds by bacterial strains are shown in Figure 5.

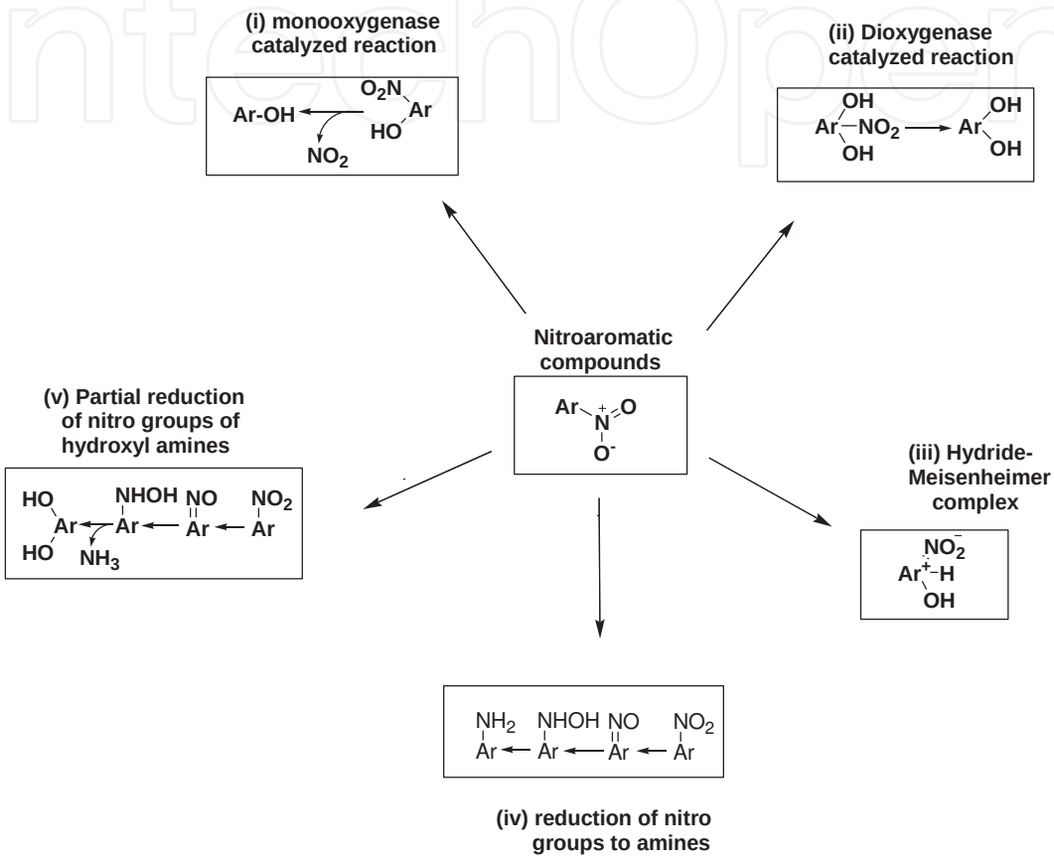

Figure 5. Different strategies of microbial remediation of nitroaromatics. (Adapted from Kulkarni and Chaudhari [49])

2.1. Anaerobic biodegradation of nitroaromatics

In this process, nitro group is reduced to nitroso derivative, hydroxyl amines, or amines by the action of nitroreductases. The degradation of most of the (poly)nitroaromatic compounds occurs only under anaerobic conditions [49, 54, 55]. The complete mineralization of nitroaromatic by a single anaerobic strain is very rare [56]. There are several reports showing that the initial step during the degradation of mono-, di-, and trinitroaromatic compounds is the reduction of nitro groups to amino groups [56–60].

2.2. Aerobic biodegradation of nitroaromatic compounds

Mono- and dinitroaromatics are mainly subjected to aerobic biodegradation and achieve to complete mineralization. Here nitroaromatics serve as source of carbon, nitrogen, and energy

for the microbe. During the past few decades, several reports came up with isolation of microbes mineralizing different nitroaromatic compound and their degradation pathway. Few of them are extensively studied and characterized. There are different strategies in the aerobic degradation of nitroaromatics [61], which is used in nature as shown in Figure 5.

2.3. Reactions catalyzed by mono-oxygenases

Mono-oxygenases are known to add single oxygen atom at a time and cause the release of nitro group. Simpson and Evans [62] reported the role of mono-oxygenase in a *Pseudomonas* sp., where 4-nitrophenol was converted to hydroquinone with the concomitant release of nitrite. Subsequently, Spain and Gibson (1991) reported accumulation of hydroquinone and release of nitrite from 4-nitrophenol by partially purified mono-oxygenase from a *Moraxella* sp.

2.4. Dioxygenase catalyzed reactions

Dioxygenases are known to add two -OH groups simultaneously on the benzene ring of nitroaromatic compounds with the release of nitro group as nitrite. This type of mechanism is reported for 2,6-dinitrotoluene biotransformation by Alcaligenes eutrophus [63]. Other examples include the degradation of 2-NT [64, 65], 3-NT [66], nitrobenzene [67], and 2,4-DNT [68].

2.5. Meisenheimer complex formation

The addition of a hydride ion to the aromatic ring of nitroaromatic compound leads to the formation of a Meisenheimer complex [27]. The complex rearomatizes after the release of nitrite anion [69].

2.6. Partial reduction of nitro groups

In this mechanism, the nitro group is partially reduced to the corresponding hydroxylamine, which upon hydrolysis yields ammonia. This mechanism was reported in Comamonas acidovorans, where 4-nitrobenzoate is converted to 4-hydroxyl-aminobenzoate [69].

2.7. Different bacterial pathways reported for the degradation of mononitrotoluenes and nitrobenzene

2.7.1. Bacterial degradation of nitrobenzene

The aerobic degradation of nitrobenzene involves two major pathways: a most commonly found partial reductive pathway and a dioxygenase catalyzed pathway (Figure 6). In the oxidative degradation of NB, degradation starts with the action of nitrobenzene-1,2-dioxygenase, which converts nitrobenzene into catechol. This catechol is further cleaved by the action of catechol 2,3-dioxygenase and degraded by the *meta* cleavage pathway. This type of pathway is reported in *Comamonas* JS 765, *Acidovorax* sp. JS42, and *Micrococcus* sp. strain SMN1.

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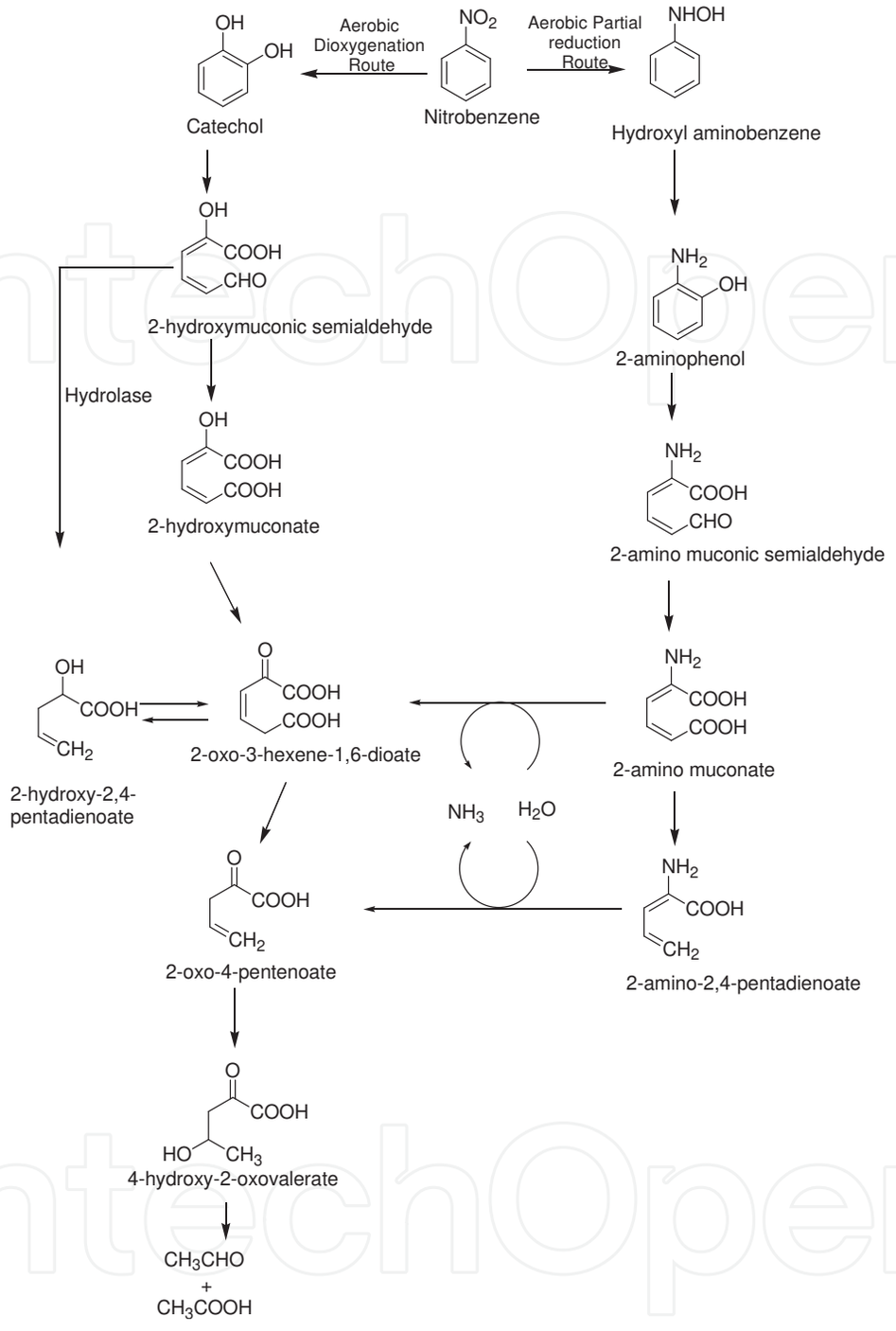


Figure 6. Degradation pathways for nitrobenzene. Aerobic dioxygenation route is reported in *Comamonas* JS765 [67]. The aerobic partial reductive pathway is from *Pseudomonas pseudoalkaligenes* JS45 [61].

2.7.2. Bacterial degradation of mononitrotoluenes

Different bacterial strains have been isolated from various sources, which can utilize nitrotoluenes as carbon source or both carbon and nitrogen source. There are several reports on different degradation pathways for mononitrotoluenes as described here.

Nitrotoluenes may be subjected to reductive pathways (formation of aminotoluenes) [70] or partial reductive pathway, wherein a nitro group is reduced to hydroxyl amino group and finally releases ammonia [71–72]. For example, during the degradation of 4-NT by *Pseudomonas* sp., initially 4-NT is converted into 4-nitrobezoic acid via the formation of 4-nitrobenzyl alcohol and 4-nitrobenzaldehyde. Then the nitro group is partially reduced to hydroxylamino derivative (rather than amino derivative), which is further converted to protocatechuate without the utilization of oxygen and release of ammonia [71, 72]. This type of mechanism was first reported for the degradation of 4-nitrobenzoate by *C. acidovorans* NBA-10 [69]. In yet another mechanism of 4-NT metabolism by *Micobacterium* sp., 4-NT was first converted to 4-hydoxyl aminotoluene followed by 6-amino-*m*-cresol. Here ammonia is released only after the ring cleavage [72].

An oxidative pathway is reported for 2-NT degradation by *Acidovorax* JS42 (Figure 7), wherein the initial oxidation of the aromatic ring takes place to form methylcatechols by simultaneous incorporation of both atoms of molecular oxygen and subsequent removal of the nitro group as nitrite by the action of a dioxygenase enzyme [64, 65, 73].

The role of mono-oxygenases and dioxygenases in the removal of nitro group from *p*-nitrophenol has also been reported from a *Pseudomonas* sp. [74–76].

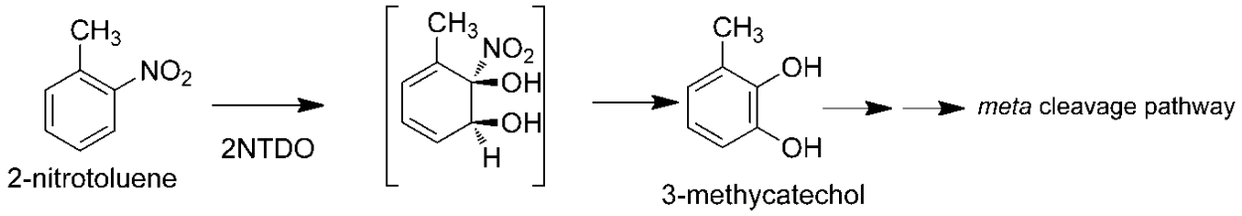


Figure 7. Degradation of 2-NT by the formation of 3-methylcatechol in *Acidovorax* sp. JS 42.

The toluene mono-oxygenase encoded by TOL plasmid oxidizes only the methyl group of 3-NT and 4-NT but not of the 2-NT [77]. Toluene dioxygenase from *Pseudomonas putida* F1 and *Pseudomonas* sp. strain JS-150 oxidatively attacks on the methyl group of 2- and 3-NT to form corresponding nitrobenzylalcohols. The enzyme, however, attacks on the aromatic ring of 4-NT to produce 2-methyl-5-nitrophenol and 3-methyl-6-nitrocatechol [81]. In both cases (either with toluene mono-oxygenase or dioxygenase as described above), the nitro group was not removed from the benzene ring and mononitrotoluene isomers did not serve as growth substrate. Degradations of monosubstituted 2-, 3-, and 4-nitrotoluenes were also reported from an adapted activated sludge system [79].

The two strains of C*omamonas* JS47 and JS46 capable of degrading 4-nitrobenzoate and 3-nitrobenzoate respectively were immobilized on alginate beads jointly and separately, and these beads were loaded in the reactor and fed to different regimes of alternating nitrobenzoate isomer or mixed nitrobenzoate isomer. Through this experiment, it was deduced that same beads containing both strains were able to recover faster from change in input composition than different beads containing different strains [80].

2.7.2.1. Bacterial degradation of 4-nitrotoluene

Two different pathways are reported in bacterial strains (as shown in Figure 8). In aerobic methyl group oxidation (*Pseudomonas* sp. strain TW3), initially 4-NT is converted to 4-nitrobenzoic acid, and then the nitro group is partially reduced to hydroxylamine derivative, which is further converted to protocatechuate without the utilization of oxygen but with concomitant release of ammonia [81]. In another mechanism, 4-NT degradation is followed by the formation of 4-hydroxylaminotoluene.

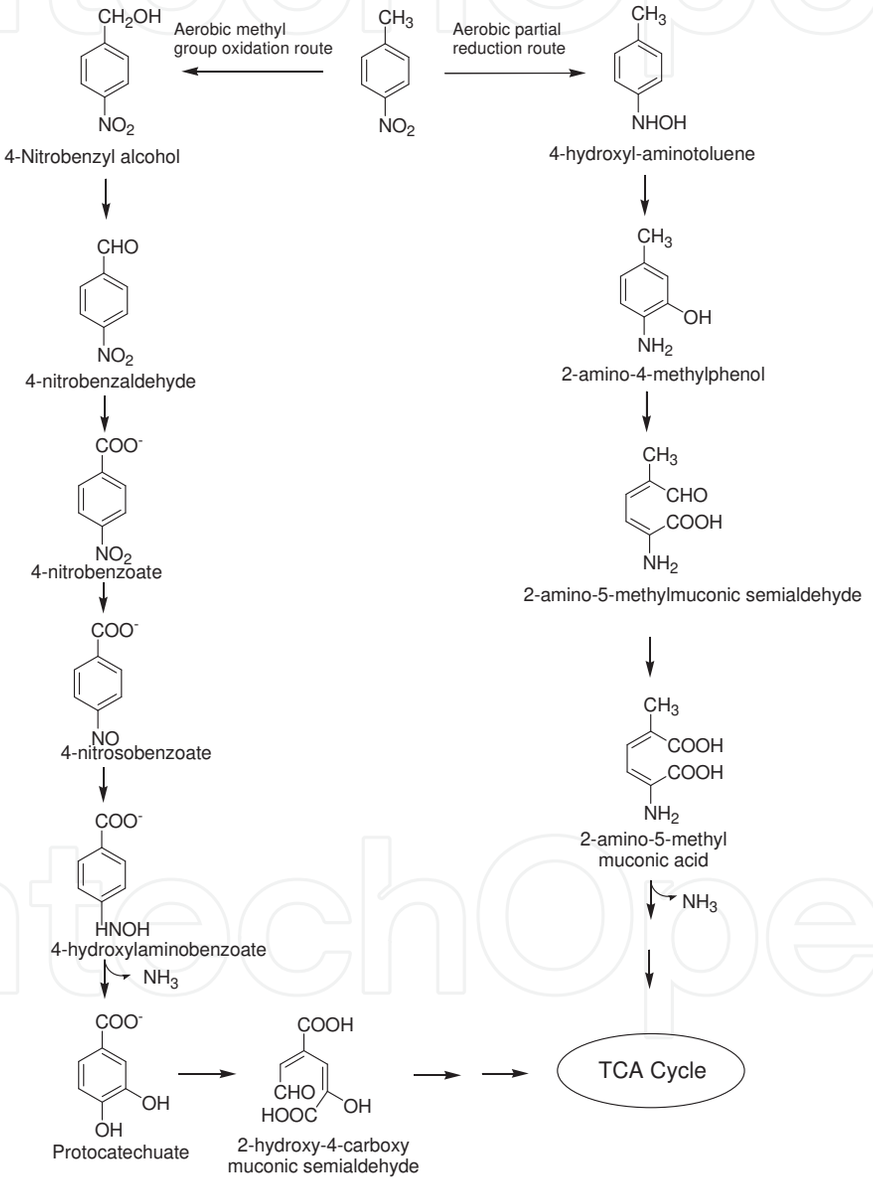


Figure 8. Degradation pathways for 4-nitrotoluene. Aerobic partial reduction rout is present in *Mycobacterium* sp. strain HL-4NT-1. Aerobic methyl group oxidation rout is reported in *Pseudomonas* strains TW3 and strain 4NT.

There are few well-characterized bacterial strains that degrades or biotransforms more than one mono-nitro compounds. 2-NT and 4-NT could transform to their corresponding amino-

toluenes and hydroxyl nitrotoluenes (pathways A and B, Figure 9) in *P. putida* OU83. Both oxidative as well as reductive attack is reported during metabolism of 3-NT [71]. Here 70% of the 3-NT was reduced to aminotoluene, whereas 30% was converted to 3-nitrophenol via the formation of 3-nitrobenzylalcohol, 3-nitrobenzaldehyde, and 3-nitrobenzoic acid. 3-Nitrophenol was further metabolized with the release of nitrite (pathway C, Figure 9).

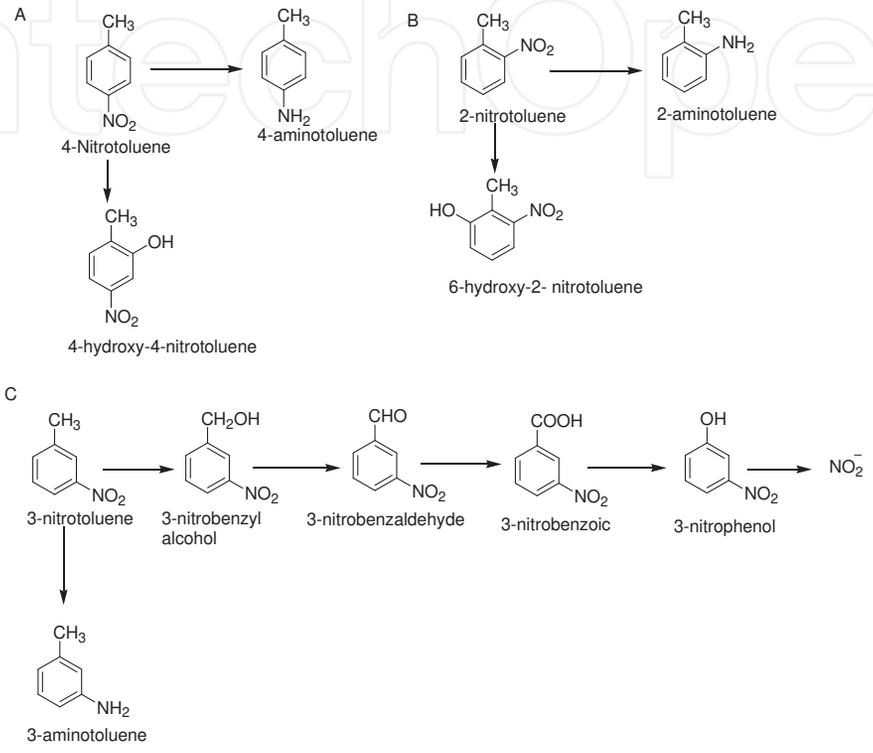


Figure 9. Degradation of mononitrotoluenes by *Pseudomonas putida* strain OU83 [82]. The strain converts 2- and 4-nitrotoluenes to corresponding aminotoluenes and hydroxyl nitrotoluenes (pathways A and B), whereas 70% of 3-NT was converted to 3-aminotoluene and 30% was degraded via the formation of the 3-nitrophenol (pathway C).

2.7.2.2. Degradation of mononitrotoluenes by Diaphorobacter sp. strain DS2

There are very few reports available on complete mineralization of mono-nitroaromatics by single bacterial strains. The isolation and characterization of three *Diaphorobacter* sp. strains DS1, DS2, and DS3, which are capable of mineralizing and utilizing 3-NT as the sole source of carbon, nitrogen, and energy, was reported by our group [66]. The mineralization of 3-NT by *Diaphorobacter* sp. strain DS2 was found by the initial reaction catalyzed by a dioxygenase with the formation of mixtures of 3- and 4-methylcatechols. These methylcatechols were further degraded by the *meta* cleavage pathway. This strain was able to degrade other compounds like 2-NT, 4-NT, nitrobenzene (NB), 2CNB, and 3CNB through an oxidative degradation route. Cloning and sequencing of first enzyme of the pathways showed the presence of a multicomponent dioxygenase, i.e., 3NTDO. This 3NTDO gene was found to be present on the genomic DNA of the strain on a 5-kb DNA stretch. Its subunits were identified as a reductase, a ferredoxin, an oxygenase large and small subunit, and a regulatory gene product [83]. Subunits

of 3NTDO were individually expressed in *E. coli* and purified by various purification techniques. To get active enzyme, all the subunits were mixed together in a certain proportion with added NADH. Its active recombinant-reconstituted enzyme showed the conversion of nitrotoluenes and nitrobenzenes to methylcatechols and catechols as measure products with the release of nitrite [84]. Some other minor products are also formed as shown in Table 1. Products other than (methyl)catechols are dead-end products and observed in the culture broth during the degradation of its corresponding substrates with the *Diaphorobacter* sp. strain DS2. This strain did not grow on any dinitrotoluenes (2,4 or 2,6), but recombinant-reconstituted 3NTDO released nitrite from 2,6-dinitrotoluene.

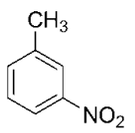
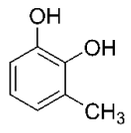
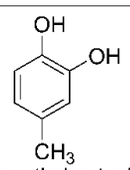
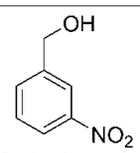
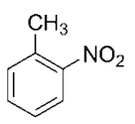
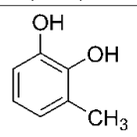
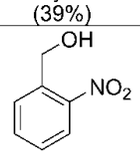
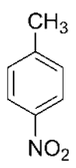
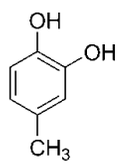
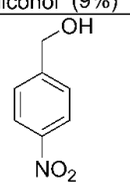
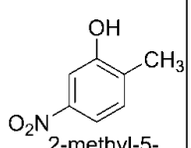
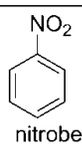
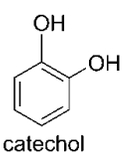
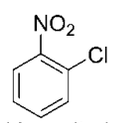
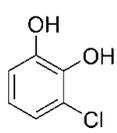
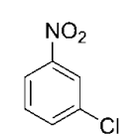
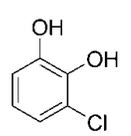
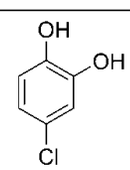
Substrate	Products formed by 3NTDO		
 3-nitrotoluene	 3-methyl catechol (54%)	 4-methyl catechol (39%)	 3-nitrobenzyl alcohol (7%)
 2-nitrotoluene	 3-methyl catechol (91%)	 2-nitrobenzyl alcohol (9%)	
 4-nitrotoluene	 4-methyl catechol (34%)	 4-nitrobenzyl alcohol (3%)	 2-methyl-5-nitrophenol (63%)
 nitrobenzene	 catechol		
 2-chloro nitrobenzene	 3-chloro catechol		
 3-chloro nitrobenzene	 3-chloro catechol (8%)	 4-chloro catechol (92%)	

Table 1. Products from the various substrates by recombinant 3NTDO.

2.8. Degradation of dinitrotoluene

2.8.1. 2,4- and 2,6-dinitrotoluene

Burkholderia sp. strain DNT and strain R34 mineralized 2,4-DNT with a nitrite removal pathway involving dioxygenase and mono-oxygenase enzyme. Here both nitro groups are not removed simultaneously but in a stepwise fashion. First, dinitrotoluene dioxygenase (DNTDO) attacks on the benzene ring converting DNT into 4-methyl-5-nitrocatachol (4M5NC) with the simultaneous removal of a nitrite group. Further, MNC mono-oxygenase removes another nitrite and converts the substrate into 2-hydroxy-5-methylquinone. These dioxygenase and mono-oxygenase enzymes have been cloned and characterized [68, 85]. This 2-hydroxy-5-methylquinone eventually leads to the formation of 2,4,5-trihydroxytoluene, a reaction that is catalyzed by HMQ reductase. Ring fission of 2,4,5-trihydroxytoluene likely occurs at position 5,6 of the aromatic ring to yield 2,4-dihydroxy-5-methyl-6-oxo-hexa-2,4-dienoic acid as ring cleavage product (Figure 10).

Nishino et al. [61, 86] isolated *Burkholderia cepacia* strain JS850 and *Hydrogenophaga paleronii* strain JS863 that were able to mineralize 2,4-DNT in the same way but degraded 2,6-DNT in a different way. When 2,4- and 2,6-DNT were used as the sole source of carbon and nitrogen together, the dioxygenation of 2,6-DNT to 3-methyl-4-nitrocatachol (3M4NC) was the initial reaction, accompanied by the release of nitrite. 3M4NC was then subjected to *meta*-ring cleavage (Figure 11) without releasing the second nitro group prior to the ring cleavage. Although 2,4-DNT-degrading strains also could convert 2,6-DNT to 3M4NC, further catabolism was halted at the point. The pathway for 2,4-DNT degradation was different from that for 2,6-DNT degradation. In the latter case, 2-hydroxy-5-nitro-6-oxohepta-2,4-dienoic acid was the first ring fission product. How 3M4NC is converted to 2-hydroxy-5-nitro-6-oxohepta-2,4-dienoic acid is unknown. In this pathway, the second nitrite group is released at the latter stages of the pathway. Position 3 methyl group appears as the determinant recognized by the initial dioxygenase to produce highly specific 3M4NC in the 2,6-DNT pathway proposed. The gene encoding this dioxygenase showed a nucleotide sequence similar to the α subunit among nitroarene dioxygenase.

The genes for the initial dioxygenases involved in 2,4-DNT and 2,6-DNT degradation are all closely related, but the enzymes are produced at low constitutive levels [27, 86]. After initial dioxygenation, the two pathways appear to diverge (Figures 10 and 11).

How does DNT degradation get affected by the presence of both isomers is important since 2,4-DNT and 2,6-DNT are produced in a 4:1 ratio [87]. These are often present together in munitions plant wastewater. Lendenmann and Spain [88] initially failed to observe the degradation of 2,4-DNT and 2,6-DNT simultaneously. Subsequently, an aerobic biofilm, initially fed with low concentrations of DNT mixture, was tested. These concentrations were then gradually increased and exhibited mineralization rates of 98% and 94% for 2,4- and 2,6-DNT, respectively. The nitrogen was released as nitrite, reflecting oxidative bacterial activity. Isomer concentration needed to be kept below inhibitory levels as high concentrations of each isomer inhibited the degradation of the other. The simultaneous degradation of 2,4- and 2,6-DNT may be unpredictable until an adapted population is established [87].

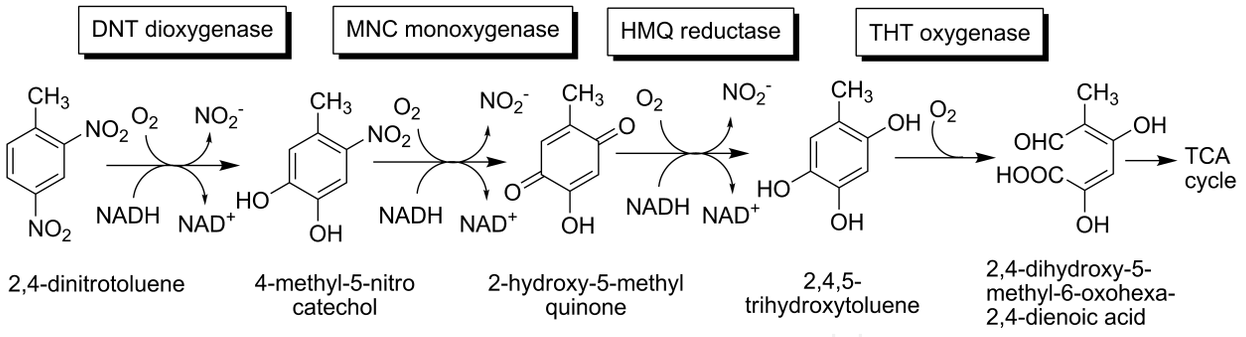


Figure 10. 2,4-DNT metabolism pathway in *Burkholderia* sp. strain DNT and strain R34.

Although nitrotoluene degraders are widely distributed at contaminated sites, the contaminants still persist for very long periods, leaving unanswered questions as to why biodegradation is ineffective to remove them. Efficient anaerobic pathways for the degradation either of mono- or dinitrotoluenes are not known, and 2,3-DNT currently does not appear to be degradable [87].

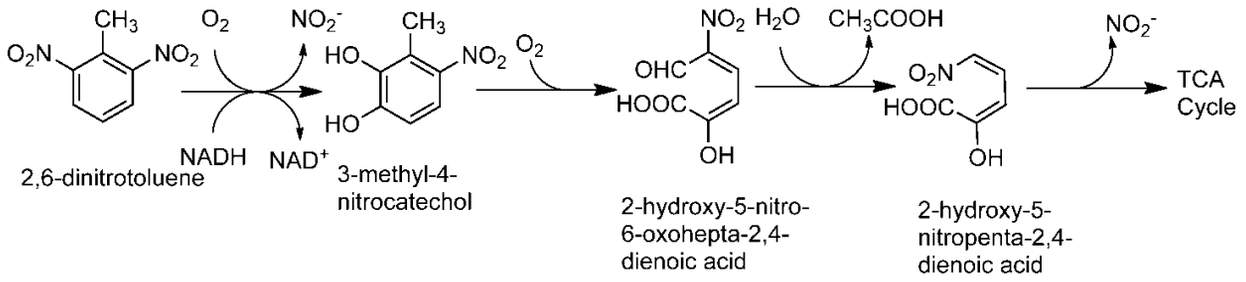


Figure 11. 2,6-DNT metabolism pathway in *Burkholderiacepacia* strain JS850 and *Hydrogenophaga paleroni* strain JS863.

Bae et al. [89] found that in an anaerobic fluidized-bed granular carbon bioreactor, 2,4 DNT can be converted completely to 2,4-diamino toluene, which subsequently mineralized in batch activated sludge reactor. Paca et al. [90] took a mixture of microbes found in the mononitrotoluene, 2,4-dintrotoluene, and 2,6-dinitrotoluene contaminated soil. These microbes were extracted and immobilized on the packing material of the packed bed reactor (PBR). Varying concentrations of 2,4-DNT and 2,6-DNT were used. In this case, two types of packing material were used out of which the reactor packed with Poraver removed 97% DNT in 11 days and the one packed with fine clay achieved the efficiency of 78%. After 20 days, the metabolites detected were 2-amino-4-nitrotoluene and 2,4-diamino toluene.

Wang et al. [91] reported that in wastewater enriched with contaminated DNT taken from Qingyang chemical industry with a DNT concentration of 3.55–95.65 mg/L, ethanol was mixed in the wastewater to act as an electron donor. The reactor in this case was made of polymethyl metacrylate containing polyurethane foams for microorganism immobilization. The microorganism used was B925. Initially, the reactors were domesticated and immobilized with

microorganism for first 10 days, and then the whole system was further operated for 140 more days, gradually increasing the concentration of 2,4 DNT. DNT was then transformed to 2-amino-4-nitrotoluene and 4-amino-2 nitrotoluene and 2,4-diaminotoluene.

2.9. Degradation of Trinitrotoluene (TNT)

Trinitrotoluene (TNT) is very difficult to degrade [87]. The three nitro groups with a nucleophilic aromatic ring structure make TNT vulnerable to reductive attack but resistant to oxygenase attack from aerobic organisms [92]. In most current reports, the reductive mechanism predominates in TNT degradation. New evidence indicates that TNT could be reduced by carbon monoxide dehydrogenase from *Clostridium thermoaceticum* [93] and by the manganese-dependent peroxidase (MnP) from the white-rot fungus *Phlebia radiata* [94]. Based on the discovery of pentaerythritoltetranitrate (PETN) reductase from *Enterobacter cloacae* PB2, French et al. [95] found that this strain could grow slowly on 2,4,6-TNT under aerobic conditions as the sole nitrogen source without the production of dinitrotoluene as an intermediate and catalyzed conversion of the TNT via a hydride–Meisenheimer complex with the nitro group released as nitrite.

Bacteria basically tends to biotransform TNT to aminonitroaromatic compounds through aerobic degradation, which in many cases turn out to be the dead-end products, and this reduced dead-end products sometimes react with themselves and form azotetranitrotoluene [96].

The removal of nitro group from the ring is essential to allow the dioxygenase to act upon it. There are very rare cases where the complete usage of TNT as the sole carbon nitrogen and energy source has also been reported. In general, most bacteria are only capable of transforming TNT to other simpler and less toxic compounds.

Due to the absence of oxygen in anaerobic processes, the formation of azonitrotoluene does not take place, thereby making the degradation through bacteria more feasible and efficient. Reduction product of TNT is very prominent in case of anaerobic process, which easily forms triaminotoluene, which is far less toxic and more soluble in water than TNT (Figures 12 and 13).

Collie et al. [116] observed the biodegradation of TNT in the liquid phase bioreactor by four different bacterial strain having pure TNT in a liquid medium. The initial concentration of TNT (70mg/L) was periodically extracted from the bioreactor for by-product identification with the help of HPLC. The bacteria used were one strain of *Enterobacter*, one strain of *Pseudomonad*, and two strains of *Alcaligenes.* Two basic intermediates, i.e., 2-amino-4,6-DNT and 4-amino-2,6 DNT, were observed with all the bacteria after 12 hours.

A bench-scale reactor by Cho and coworkers [114] used *P. putida* HK6 (collected from RDX-contaminated soils). This was used for the degradation of several nitroaromatic compounds simultaneously in one go, i.e., TNT–RDX–atrazine–simazine (TRAS). Cells were grown in a liquid media composed of 30–100 mg TNT, 5–15 mg RDX, 20–50 mg atrazine, and 5–15 mg simazine and other basal salts like K_2HPO_4, NaH_2PO_4, $MgSO_4$ $7H_2O$, $CaCl_2$ $2H_2O$, $FeCl_3$ $6H_2O$ in appropriate quantities, and subsequent experiments on the utilization of nitroaromatic compounds were carried out using a 2.5-L bottom driving type bench-scale reactor with a water condenser at 5°C and operated at 30°C at 150 rpm. A 10% inocula of test culture was

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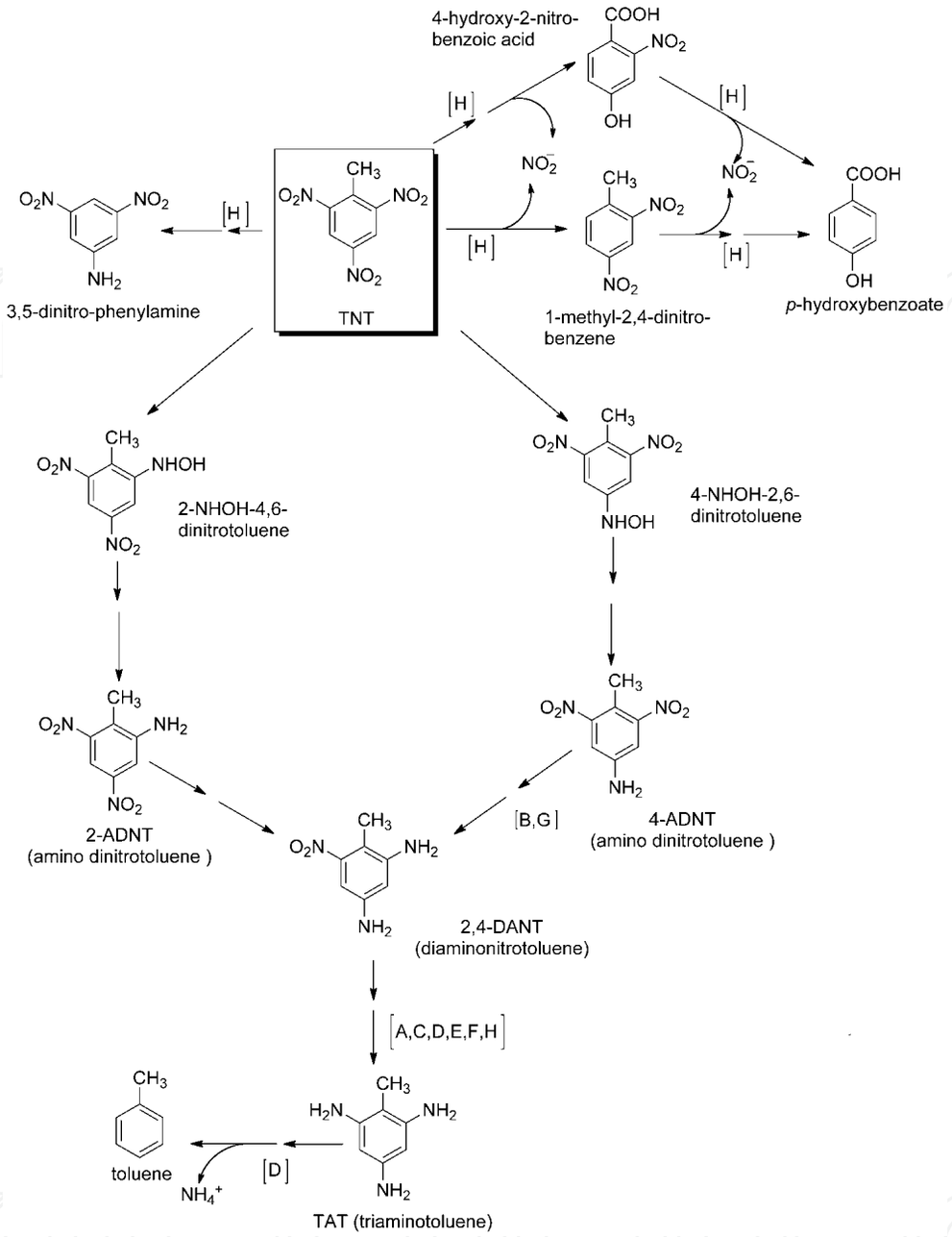

Figure 12. Different reductive pathways in different bacteria: (*A*) *Clostridium acetobutylicum, Escherichia coli*, *Lactobacillus* sp. [97]; (*B*) *Clostridium bifermentans* CYS-1 [98]; (*C*) *Clostridium bifermentans* LJP-1 [99]; (*D*) *Disulphovibriosp* strain B [100]; (*E*) *Disulphovibrio* sp. [101]; (*F*) *Disulphovibrio* sp. [102]; (*G*) *Methanococcus* sp. strain B [103]; (*H*) *Veillonella alkalescens* [57].

grown with nitroaromatic compound, and the turbidity observed showed that the bacteria *P. putida* HK6 was able to degrade 100 mg/l TNT, 15 mg/l RDX, 50 mg/l atrazine, and simazine in 4, 24, 2, and 4 days after incubation, respectively. In this experiment, it is noteworthy that the presence of Tween-80 in the culture led to the complete degradation of TRAS compounds, whose otherwise partial degradation was TNT (80%), RDX (35%), and simazine (78%) during the incubation period [114].

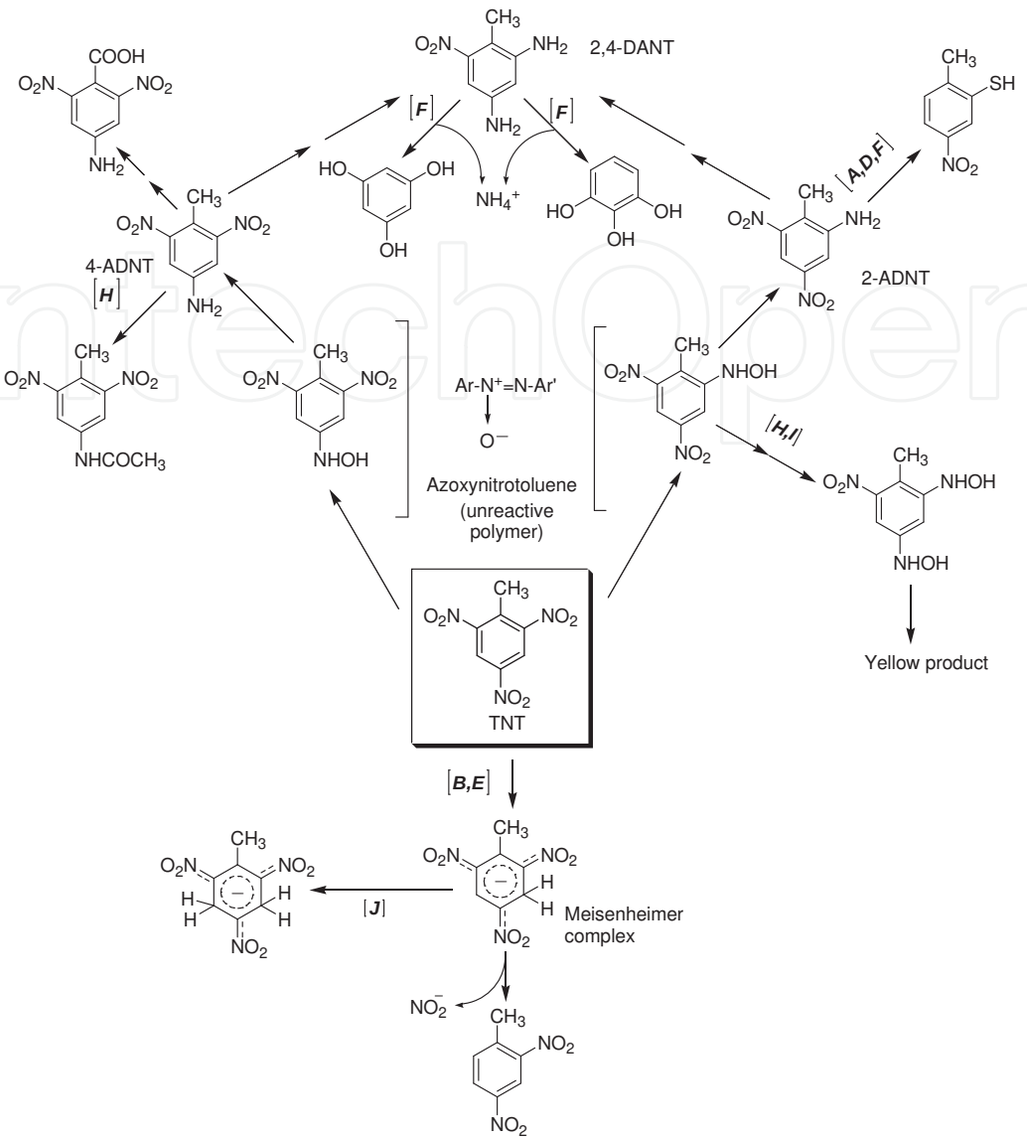

Figure 13. Complete representation of the aerobic pathway of TNT conversion to its products performed by the group of bacteria shown in the figure: (*A*) *Bacillus* sp., *Pseudomonas aeruginosa, Staphylococcus* sp. [104]; (*B*) *Enterobacter cloacae* PB2 [105]; (*C*) *Enterobacter* sp. [89]; (*D*) *Pseudomonas aeruginosa* MA101 [106]; (*E*) *Pseudomonas florescence* [107]; (*F*) *Pseudomonas florescence* B3468 [108]; (*G*) *Pseudomonas florescence* [109]; (*H*) *Pseudomonas pseudoalcaligenes* [110]; (*I*) *Rhodococcus erythropolis* [111]; (*J*) *Serratia marcensens* [112].

Similarly, the degradation of some other nitroaromatic compounds was reported on the basis of field trials. A highly active microbial consortium was chosen by Oh et al. [115] for field trials to degrade the wastewater samples having 4,6-dinitro-*ortho*-cresol (DNOC) [116]. Fixed bed column reactors were employed to increase the volume density of active biomass and to degrade DNOC in wastewater stream. In this case, glass bead matrix was used to immobilize the bacterial colonies. The mixed bacterial culture CDNOC1-3 metabolizes DNOC with concomitant liberation of nitrate. The efficiency of the bioreactor was 86%. This was substantially higher over a batch culture mode (60–65%). The following schematic diagram (Figure 14) shows the fixed bed column reactor system [116].

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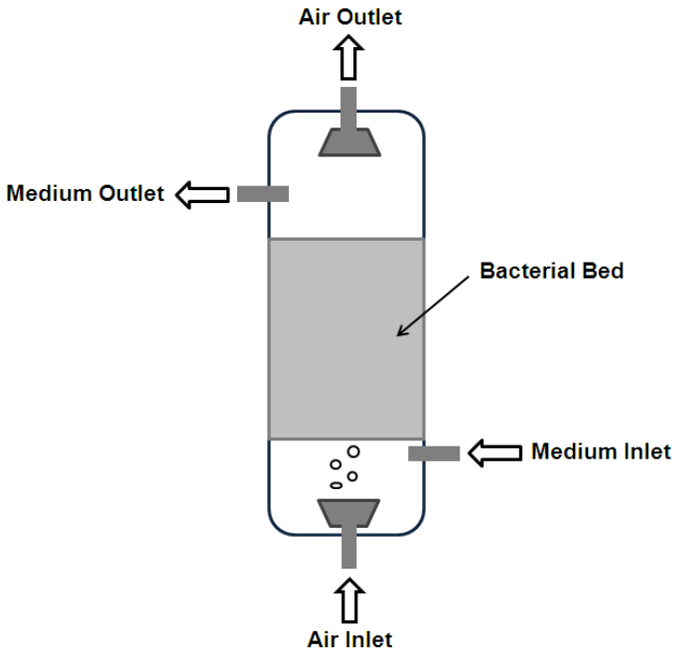

Figure 14. Schematic representation of the reactor used for the degradation of 4-nitrobenzoate and 4-aminobenzoate by *B. cepacia* PB4 in the abovementioned study [59].

A similar kind of experiment was performed in year 1999 in which *B. cepacia* strain PB4 was isolated from 4-aminobenzoate. This also possessed the capability to degrade 4-nitrobenzoate. Thus, considering both classes of the contamination to be toxic and mutagenic, an efficient strategy of decontamination was applied because *B. cepacia* was able to use both as the sole source of carbon, nitrogen, and energy. In order to prevent toxic effects, these compounds were supplied in lower concentration, i.e., 10–100 ppm and also in order to increase the efficiency of the procedure at such low concentration the degradative bacteria was immobilized on porous diatomaceous celite. This degradation was carried out in a packed bed reactor (PBR). The bioreactor consisted of a glass tube (296 × 41 mm) filled with 150 mm packed bed of celite-grade R-633 or R-635, which was placed at 30°C. It was shown that the nitroaromatic and aromatic amino compounds, which are otherwise unlikely to degrade together if present in any affected area, had been degraded simultaneously by single microorganism supply.

The eventual objective of all the biochemical and molecular characterization of the bacterial degradation of pollutants is to develop strains, which could be used in the bio remediation process. In this respect, another good field trial experiment was described by Labana et al. [117, 118] with bacterial strain *Arthrobacter protophormiae* RKJ100. The result clearly showed that the disappearance of the nitroaromatic pollutants.

Similarly, *Pseudomonas* sp. ST53 was also used as a microbe to degrade TNT and other explosives, but it is best suited on land and water only when the contamination is low [119]. Qureshi et al. [120] reported a bacterial strain *Arthrobacter* sp. HPC1223, which was capable of degrading 2,4,6-trinitrophenol prominently. This also poses the capability to degrade dinitrophenol and mononitrophenol showing broad substrate specificity.

3. Evolution of genes for nitroaromatic degradation

Nitroaromatics are recent compounds present in the environment and bacterial strains that adapted themselves for the removal of these compounds. This was possible only through evolution of its degradation system at a genetic level.

3.1. Modes of gene evolution

3.1.1. Mutational drift

Substrate profile of an existing enzyme may be altered by point mutations in its corresponding genes [124]. The reasons for changes in primary DNA sequences are slippage of DNA polymerase while replications, erroneous DNA repair, and gene conversion [122]. However, results of these changes are relatively smaller. These alone cannot be accountable for adaptation to the new environment by bacteria [123, 121].

3.1.2. Genetic rearrangement within a cell

The rearrangement of genes for the development of new pathway may take place by the help of cells own recombination system. Gene segments can be exchanged between two positions flanked by homologous sequences, insertion elements, transposons, and even sequence identities of four base pairs are sufficient to facilitate this process [122].

3.1.3. Horizontal gene transfer

Horizontal gene transfer is reported as the main source of evolution of pathways in bacteria [123, 124]. Sequencing results of genomes from different bacterial strains have revealed the presence of acquired genes in mosaic like fashion throughout bacterial genomes. Their presence varies from almost negligible (*Rickettsia prowazekii* and *Mycobacterium genitalium*) to about 17% (in *Synechocystis* strain) [123]. Plasmids also play a major role in carrying catabolic genes during horizontal gene transfer [125]. Transposons are also known to facilitate the catabolic gene transfer processes [126].

3.2. Nitroaromatic degradation pathway as a role model for study the gene evolution

A role model to study evolution of microbial pathways is to study the degradation of nitroaromatic compounds in different bacteria.

Nitroaromatic compounds are relatively new to the environment, but bacterial systems have already evolved the ability to metabolize them. This cannot be possible only by spontaneous, independent evolution of several new enzymes in a single bacterium. Horizontal gene transfer has to play a key role in combination with the mutagenesis of the existing enzymes to facilitate rapid evolution of new pathways. Evolution of diverse pathways for the degradation of different nitroaromatics thus stands testament to this.

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A good example of this is the evolution of chloronitrobenzene dioxygenase system from a chloronitrobenzene degrading strain *Pseudomonas stutzeri* [127]. It has several insertion sequences embedded between the gene clusters, which proves such involvement in its evolution. In its dioxygenase enzyme system, reductase and ferredoxin seems to have come from different origins because its reductase and ferredoxin share maximum identity with anthranilate dioxygenase, which is a type IV oxygenase, whereas its oxygenase subunits show a maximum identity with nag (naphthalene degradation gene) and nitroarene dioxygenase, which falls under type III oxygenase systems. This enzyme system thus best illustrates the evolution of catabolic genes best because its upper pathway enzymes seem to have originated from a nitroarene degradation pathway and its lower pathway genes have evolved from some chloroaromatic compound degradation pathway. The genes responsible for these pathways are present in a patchwork like assembly in *P. stutzeri* [127]. The presence of insertion elements in the gene cluster confirms its role in the formation of a modular assembly and its role in evolution of the gene cluster.

Another example is the origin of 2,4-DNT degradation pathway in *B. cepacia* R34 where enzymes for the degradation pathway have originated from at least three different sources [128]. The first enzyme in the degradation pathway (DNTDO), which removes the first nitrite from 2,4-DNT, seems to have originated from naphthalene degradation pathway like in *Ralstonia* sp. strain U2. [129]. However, 4-methyl-5-nitrocatechol mono-oxygenase, which facilitates the removal of the second nitrite, appears to be derived from a pathway for degradation for chloroaromatic compounds [128]. The last enzyme of the pathway could have originated from a gene cluster for amino acid degradation. This is known as progressive compaction of the genes. However, the presence of ORFs without any known role in 2,4-DNT degradation and truncated transposons in the regions suggests that compaction is probably in an intermediate stage in the evolution of such an optimal system with the genetic materials from different bacterial origin.

A good example of similar type of evolution is reported in diphenylamine degrader *Burkholderia* sp. strain JS665 [130]. In this pathway, diphenylamine is converted to catechol and aniline. An analysis of this sequence of diphenylamine degrading enzyme system showed that it has evolved by recruiting two pathway enzymes, one of which is from dioxygenase and the other is from nitroaniline degradation pathway enzymes in a much more recent evolution event [127].

Another example of evolution of genes for nitrotoluenes degradation is the evolution of 3NTDO in *Diaphorobacter* sp. strain DS2. Five complete ORFs were identified by probable ORF finding program and by homology to polypeptide sequences from several previously reported multicomponent dioxygenase systems. The predicted translation products from ORFs were designated as a putative regulatory protein, a ferredoxin reductase subunit, a ferredoxin subunit, an oxygenase large, and a small subunit based on their homology. A 571-bp DNA stretch was present in between reductase and ferredoxin subunits. In its gene structure, the regulatory protein is divergently transcribed from the other four ORFs. The organization of gene cluster and its similarity with other known dioxygenases is shown in Figure 15.

It has been suggested in several reports that *Ralstonia* sp. strain U2 [129] is the progenitor of all the nitroarene dioxygenases because it has the entire functional gene in its gene assembly.

This seems true for the 3NTDO as well because the sequence present in between the reductase and the ferredoxin is truncated into parts of two functional ORFs present in the *Ralstonia* sp. strain U2. The regulatory protein sequence of 3NTDO (MntR) differs only at three amino acid positions from NagR of *Ralstonia* sp. strain U2 out of which two are uniquely present in strain DS2 only.

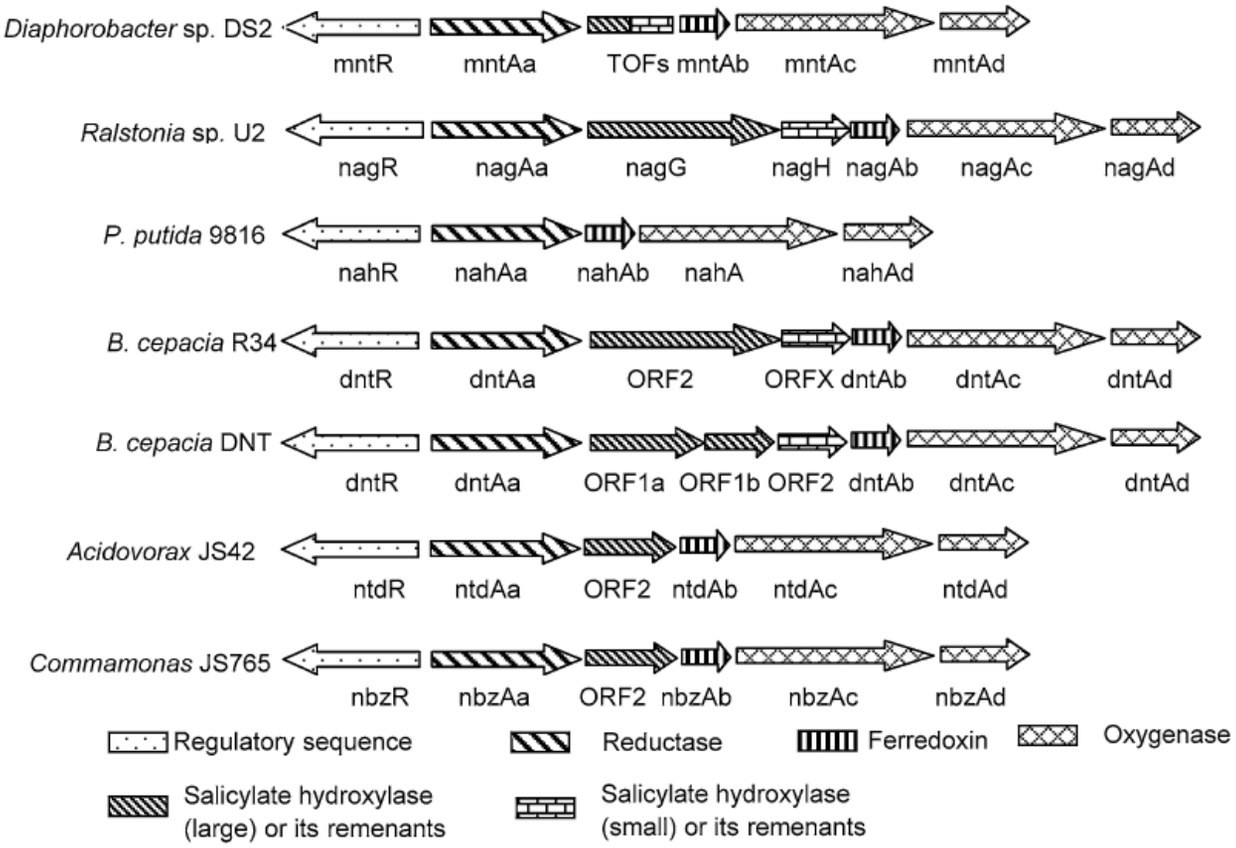

Figure 15. Comparison of gene arrangements in various multicomponent dioxygenases.

The different components (reductase, ferredoxin, and oxygenase) of 3NTDO show different levels of sequence identity with components from similar multicomponent enzyme systems of different organisms. Its reductase subunit (MntAa) shares a high amino acid sequence identity with those of DNTDO from *B. cepacia* [128] and NDO of *Ralstonia* sp. strain U2 (99%) [129], but its ferredoxin subunit (MntAb) is 100% identical to the ferredoxin of dinitrotoluene dioxygenase from *Burkholderia* sp. strain DNT and *Burkholderia* sp. strain R34. Its large oxygenase subunit (MntAc) showed more identity with chloronitrobenzene dioxygenase (CnbAc, 96%) from *P. stutzeri* ZWLR2-1, whereas the small oxygenase subunit (MntAd) showed more identity with PahAd of *Comamonas testosteroni* (96%) and NTDO from *Acidovorax* sp. strain JS42. It is known that oxygenase large subunit controls substrate specificity. If we compare important active site residues in oxygenase large subunit (MntAc) of strain DS2 with well-characterized oxygenase systems, it contains amino acid combinations of other systems, in which the sequence retains His293, which is present in NDO system of *P. putida* 9816-4, *Ralstonia* U2, *C. testosteroni* H, and *Burkholderia* sp. C3. Position 350 is occupied by Valine, which

is reported in the DNTDO of *Burkholderia* sp. strain R34. Thus, the above facts seem to indicate that 3NTDO gene in *Diaphorobacter* sp. strain DS2 came through a horizontal gene transfer from ancestors common to strains like *Ralstonia* U2 or *Burkholderia* sp. strain R34, and then its catalytic subunit has been diversely evolved to degrade other nitroaromatic compounds.

The mechanism by which enzymes for the degradation of synthetic compounds have evolved so rapidly still cannot be explained only by horizontal gene transfer and mutations. It can be explained in part by the term promiscuity. Promiscuity refers to the ability of a protein to perform dual functions using same active site [131, 132]. Protein evolution toward a new function based on promiscuity involves transition of an existing specialized enzyme to a generalized intermediate enzyme and then into a new specialized enzyme (Figure 16). A good example of this is transcriptional regulator found in nitroarene dioxygenases [133].

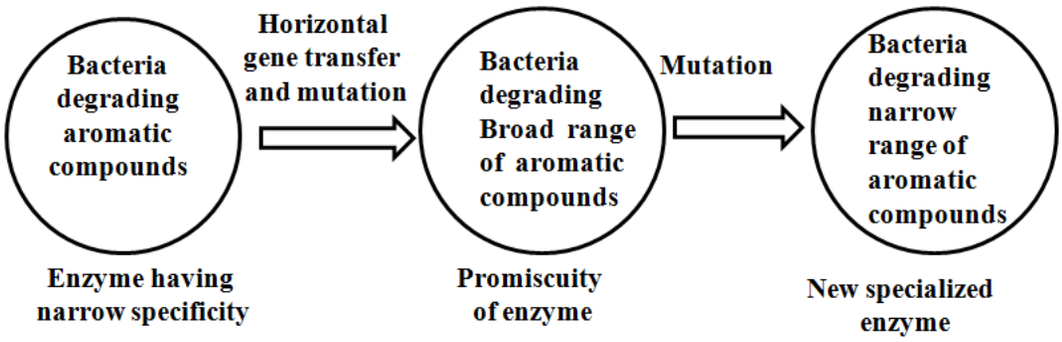

Figure 16. Evolution of new specialized enzymes from existing one. (Modified from Ju et al. [134])

The correct functioning of a pathway depends not only on having enzymes with appropriate catalytic activity but also on regulators, which control the expression of the catabolic genes in response to the compounds to be degraded. For example, ntdR, which controls 2NTDO expression in *Acidovorax* JS42, is supposed to have evolved from an ancestral nagR regulator of naphthalene degradation pathway present in *Ralstonia* U2. NtdR differs only by five amino acids with nagR. Ju et al. [134] showed how ntdR like regulator could be created from nagR by making mutations at each of the five positions separately and in combinations in a stepwise manner. They also showed that each mutation broadened the effectors range in a stepwise manner without losing the original activity. Both NagR and NtdR can activate transcription in the presence of salicylate, which is a natural inducer of naphthalene degradation genes in strain *Ralstonia* sp. U2, but ntdR could have gained a broader effector specificity to recognize several nitroaromatic compounds too [134]. Hence, the evolution of the regulatory system of the 2NTDO is in an intermediate stage because it can be induced in response to several nonmetabolizable compounds. Thus, the selection of ntdR variant with high specificity for 2NT with loss of specificity for salicylate would enable the identification of mutations that can lead to the specialized transcription factor from an intermediate stage. Another regulator was reported by Singh et al. [83], where the regulatory protein sequence of 3NTDO (MntR) differs only at three amino acid positions from NagR of *Ralstonia* sp. strain U2, out of which two are uniquely present in strain DS2 only.

It can be concluded that the gene evolution in these dioxygenase systems cannot be explained by considering only one mode of evolution. All the modes of evolution (like horizontal gene transfer, selective mutation, and promiscuity) are responsible for the evolution of a dioxygenase system [133, 135]. Further, the presence of truncated ORFs (which is not required for enzyme activity) reveals that gene evolution is in an intermediate stage of the so-called progressive compaction of the genes.

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