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Nitroaromatic Compounds, from Synthesis to Biodegradation

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INTRODUCTION

Nitroaromatic compounds are among the largest and most important groups of industrial chemicals in use today. These compounds are organic molecules that consist of at least one nitro group (-NO_2) attached to an aromatic ring. The vast majority are synthetic, although several biologically produced nitroaromatic compounds have been identified. The strong electronegativity of the nitro group stems from the combined action of the two electron-deficient oxygen atoms bonded to the partially positive nitrogen atom. When attached to a benzene ring, the nitro group is able to delocalize π-electrons of the ring to satisfy its own charge deficiency. This not only provides charge to the molecule but also imparts unique properties that make the nitro group an important functional group in chemical syntheses. The nitro group is strongly deactivating toward electrophilic aromatic substitution of the benzene ring. Both the conjugation state and resonance properties of nitro groups attached to aromatic rings result in partially positive charges at *ortho* and *para* positions that act to repel electrophiles, and as a consequence, attacks are directed toward the open *meta* positions. Furthermore, when aromatic compounds with multiple nitro groups react with electrophiles, stable Meisenheimer complexes can be formed. These characteristics contribute to the stability and recalcitrance to degradation of this class of chemicals.

Over the last several years, numerous review articles have specifically addressed the toxicity and mutagenicity of nitroaromatic compounds (117, 140, 152, 162), the biosynthesis of nitro compounds (205), and the biodegradation of nitroaromatic compounds (132, 135, 180, 181, 188). Here we present an integrated review of the chemical and biological syntheses of nitroaromatic compounds and our current understanding of bacterial degradation of these toxic and recalcitrant chemicals.

SYNTHETIC NITROAROMATIC COMPOUNDS

Nitration is the main reaction used to synthesize nitroaromatic compounds. Nitronium ions (NO_2^+) are generated in a mixed-acid reaction of sulfuric and nitric acids and then added onto aromatic substrates via electrophilic substitution (11). In this fashion, benzene, toluene, and phenol are converted into nitrobenzene, nitrotoluenes, and nitrophenols, the simplest of all nitroaromatic compounds. Conditions can be modified to direct nitration to the *ortho*, *meta*, or *para* position. In the Zinke nitration, phenols or cresols react with sodium nitrite to replace bromines with a nitro group (156–158). Nitration can also be tailored to multiple substitutions on a single molecule. In the Wolffenstein-Böters reaction, nitration of benzene with nitrous acid and mercury nitrate results in the production of 1,3,5-trinitrobenzene (35).

The unique chemistry of the nitro group has led to the use of several nitroaromatic compounds in high-energy explosives (Fig. 1). In this oxidation state (+III), the nitrogen atom readily accepts electrons and thereby allows nitroarene explosives to act as self-oxidants. As a result, energy is rapidly released from these compounds when an explosive charge is detonated (171). Picric acid (1,3,5-trinitrophenol) was first prepared in 1771 as a yellow dye for fabrics (108) and has been used in explosive shells. However, the corrosiveness of picric acid, its reactivity with metals to form shock-sensitive salts, and its incomplete detonation led to its eventual disuse. In contrast to picric acid, 2,4,6-trinitrotoluene (TNT) (Fig. 1) is chemically stable and insensitive to impact (138). Although TNT was widely manufactured by sequential nitration of toluene and

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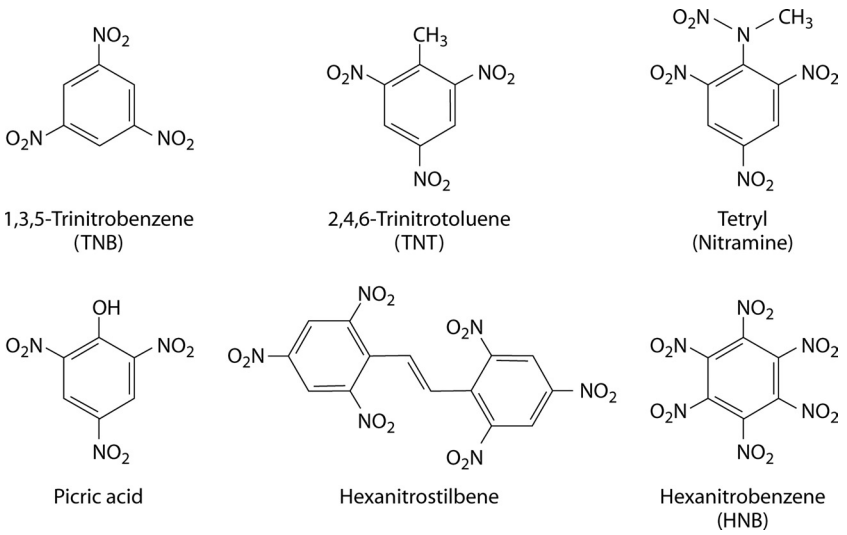


FIG. 1. Nitroaromatic explosives.

was extensively used in both World Wars, it is no longer produced in North America due to problems of environmental contamination and persistence at manufacturing sites. TNT is still found as a major component of many composite explosives that include chemicals such as aluminum, barium nitrate, or other explosives, such as the heterocyclic nitroaromatic compounds cyclotrimethylenetrinitramine (RDX) and cyclotetramethylenetetranitramine (HMX) (108, 138).

TNT also serves as the starting point for the synthesis of other nitroaromatic explosive compounds. Two molecules of TNT can be fused together by oxidative coupling of the methyl groups to produce hexanitrostilbene (Fig. 1), which has increased thermal stability (138). Elimination of the methyl group of TNT can also be directed to produce 1,3,5-trinitrobenzene (TNB) (Fig. 1), which is a higher-energy explosive with decreased shock sensitivity (138). The explosive properties of TNB can be enhanced further by partial reduction of the nitro groups followed by nitration of the open positions of the benzene ring and then reoxidation to form hexanitrobenzene (HNB) (Fig. 1). However, the hygroscopic nature of HNB results in its hydrolysis into pentanitrophenol, tetranitroresorcinol, and trinitrophloroglucinol, thereby limiting its application in munitions (108, 138).

In addition to explosives, many commonly used industrial and consumer products are produced using nitroaromatic compounds as starting materials. Nitrobenzene, nitrotoluenes, nitrophenols, and their halogenated derivatives serve as starting compounds in the production of a wide variety of pesticides (Fig. 2). Nitrophenols are used in the synthesis of compounds such as carbofuran (177), parathion (47), fluorodifen (76), nitrofen, and bifenox (203). Dinitrophenols have been used in the production of all categories of pesticides (ovicides, insecticides, herbicides, fungicides, etc.) and include compounds such as 2,5-dinitro-*o*-cresol, dinoseb, and binapacryl (203).

Many pharmaceuticals also have their chemical origins in nitroaromatic compounds. Substituted nitrobenzenes and nitropyridines are used to create a diverse collection of indoles, which are bioactive components not only of drugs but also of agrochemicals (33). Derivatives of phenothiazines, a large class of drugs with antipsychotic properties, can be synthesized using nitrobenzene or halonitrobenzenes (54, 170). Chloronitrobenzenes are feedstocks used to create new derivatives of anpirtoline, a nonopioid analgesic (155). Synthesis of lidocaine (a local anesthetic) is a classic organic chemistry laboratory exercise that starts with the reduction of 2,6-dimethylnitrobenzene to 2,6-xylidine. Paracetamol, also known as acetaminophen, which is sold as an over-the-counter analgesic and antipyretic, is produced in a one-step reductive acetamidation of *p*-nitrophenol (8).

Aromatic amines, one of the largest groups of feedstocks used by the chemical industry, are produced by catalytic reduction of nitroaromatic compounds. Aniline, with a worldwide consumption of approximately 3 million tons in 2003, is produced on an industrial scale in a two-stage process in which benzene is first nitrated and purified to yield nitrobenzene and then hydrogenated using a metal catalyst and hydrogen gas (208). Anilines not only are used to synthesize drugs, pesticides, and explosives but also are the fundamental building blocks in products such as polyurethane foams, rubber, azo dyes, photographic chemicals, and varnishes (196).

NATURALLY OCCURRING NITROAROMATIC COMPOUNDS

Nitroaromatic compounds can form naturally in both atmospheric and aqueous environments. In urban settings, hydrocarbons released from natural combustion processes and the incomplete combustion of fossil fuels serve as substrates for nitration with atmospheric nitrogen dioxide. Through a hydroxy radical-initiated mechanism, nitrobenzene, 3-nitrotoluene, 1- and 2-nitronaphthalene, 3-nitrobiphenyl, and mixtures of many other nitro-polyaromatic hydrocarbons (nitro-PAHs) can be produced (3, 4, 128, 152, 165). In aqueous environments, sunlight catalyzes nitration and halogenation reactions of naturally occurring or anthropogenic compounds in a similar fashion. Solar irradiation of dissolved organics, metal spe-

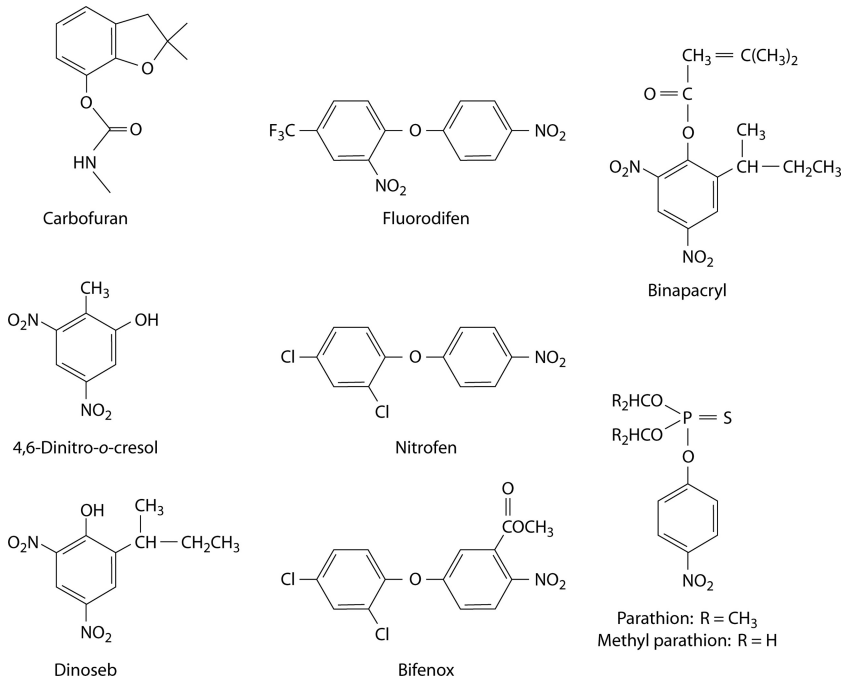


FIG. 2. Pesticides synthesized from nitrophenols.

cies, nitrate, or nitrite can generate hydroxyl radicals, which then serve as catalysts for both halogenation and nitration reactions of organic compounds. Irradiation of seawater containing phenol resulted in the production of not only 2- and 4-nitrophenol but also chlorophenols and bromophenols (17). Nitration also occurs in the atmospheric aqueous phase, producing nitro-PAHs and nitrophenols, which can then be deposited terrestrially by rain or snow (198, 199).

Although the vast majority of nitroaromatic compounds are manufactured chemicals, they have also been discovered as natural products from a variety of bacteria, fungi, and plants (recently reviewed in reference 205). Members of the genus *Streptomyces* are known to produce a wide variety of antibiotics, including those with a nitroaromatic component (Fig. 3 and 4). Perhaps the best-known nitroaromatic antibiotic is chloramphenicol (originally named chloromycetin), produced by

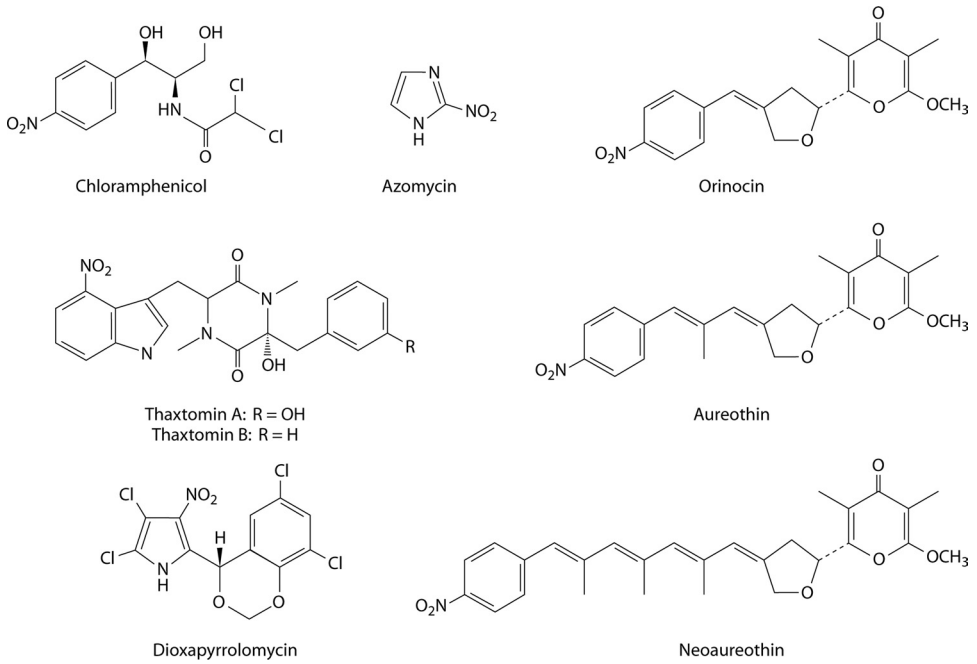


FIG. 3. Nitroaromatic antibiotics produced by bacteria of the genus *Streptomyces*.

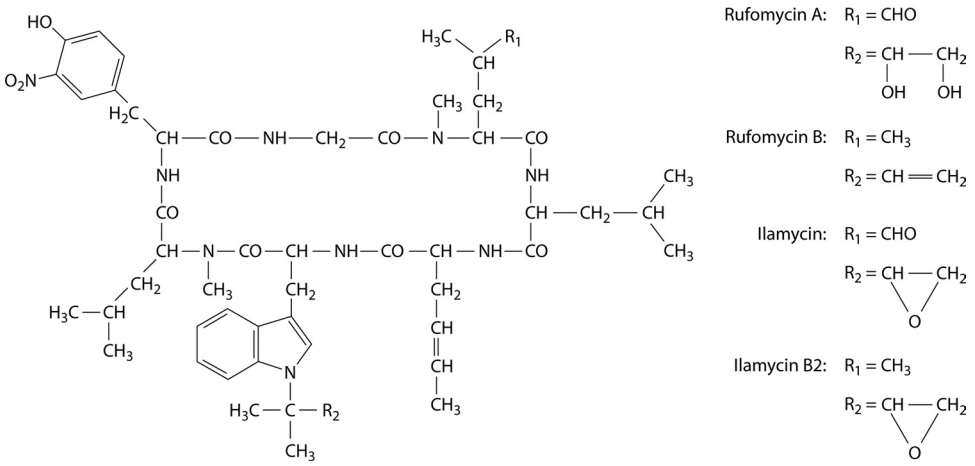


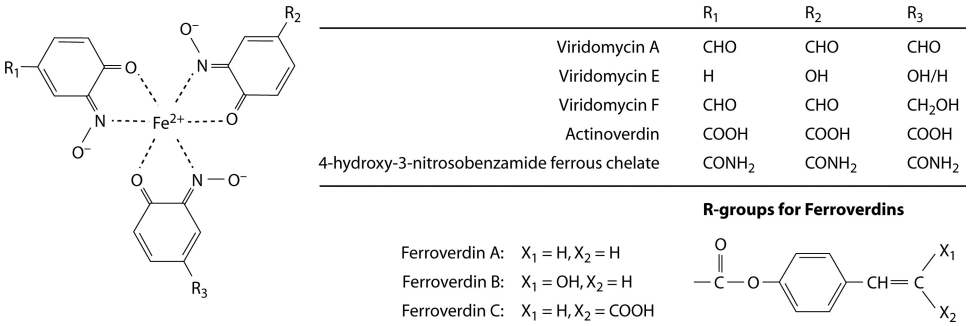
FIG. 4. Rufomycins and ilamycins produced by bacteria of the genus *Streptomyces*.

Streptomyces venezuelae (42, 53, 178). Other nitro group-containing antibiotics produced by streptomycetes include aureothin, a polyketide from *Streptomyces thioluteus* (18, 77, 118); neoaureothin (spectinabilin) and orinocin, polyketides from *Streptomyces spectabilis* and *Streptomyces orinoci* (93, 122, 195), respectively; azomycin, a nitroimidazole from *Streptomyces eurocidicus* (137); dioxapyrrolomycin, a chloro-nitro compound from *Streptomyces fumanus* (19, 22); thaxtomins, structurally diverse nitro-dipeptides produced by several species of *Streptomyces* that are pathogens of plant tubers (98, 116); rufomycins (Fig. 4), which are cyclic heptapeptides from *Streptomyces atratus*; and the structurally similar ilamycins, from *Streptomyces islandicus* (193), which contain a nitro group at the *meta* position of tyrosine (49, 174).

Streptomyces strains have also been found to produce a variety of siderophores that have *o*-nitrosophenol with different functional groups attached at the *para* position (Fig. 5). Three molecules chelate one ferrous iron atom through the oxygen of the phenol and the nitrogen of the nitroso group, resulting in green coloration. Ferroverdin A was originally isolated from a *Streptomyces* strain and contains three substituted *p*-vinylphenyl-3-nitroso-4-hydroxybenzoate groups (5, 21). Ferroverdin B (two *p*-vinylphenyl-3-nitroso-4-hydroxybenzoate and one hydroxy *p*-vinylphenyl-3-nitroso-4-hydroxybenzoate functional group) and ferroverdin C (two *p*-vinylphenyl-3-nitroso-4-hydroxybenzoate and one carboxylic acid *p*-vinylphenyl-3-nitroso-4-hydroxybenzoate functional group) were later discovered from *Streptomyces* sp. WK-5344 (189).

Several other related siderophores contain functional groups other than the *p*-vinylphenyl substitution. Viridomycin A, from *Streptomyces viridaris* 1671 (212) and several *Streptomyces griseus* strains (101), contains an aldehyde at the *para* position. *Streptomyces griseus* strains also produce actinoviridin, which has a carboxylic acid substitution (101). Viridomycin F, from *Streptomyces* sp. K96-0188 (136), is composed of two aldehyde-substituted molecules and a hydroxylated methyl as the third, while viridomycin E (*Streptomyces griseus*) is a mixture of molecules with an alcohol at the *para* position and those with no substitutions (101). *Streptomyces murayamaensis* was found to produce a compound with a carbonylamine substitution (4-hydroxy-3-nitrosobenzamide ferrous chelate) (29).

In addition to chelating ferrous iron, some of the above siderophores have additional bioactivities. Ferroverdins A, B, and C were found to be inhibitors of human cholesteryl ester transfer protein (194), an important mediator of cholesterol levels and a contributing factor to high blood pressure and



	R_1	R_2	R_3
Viridomycin A	CHO	CHO	CHO
Viridomycin E	H	OH	OH/H
Viridomycin F	CHO	CHO	CH_2OH
Actinoverdin	COOH	COOH	COOH
4-hydroxy-3-nitrosobenzamide ferrous chelate	$CONH_2$	$CONH_2$	$CONH_2$

R-groups for Ferroverdins

FIG. 5. Nitroaromatic siderophores produced by bacteria of the genus *Streptomyces*. Ferroverdins are similar to viridomycins but have three variously substituted *p*-vinylphenyl-3-nitroso-4-hydroxybenzoate groups bound to the Fe^{2+} (R groups are shown). Three *p*-vinylphenyl-3-nitroso-4-hydroxybenzoate groups are bound in ferroverdin A. In contrast, ferroverdins B and C are composed of two molecules of *p*-vinylphenyl-3-nitroso-4-hydroxybenzoate, with a hydroxy (ferroverdin B) or carboxylic acid (ferroverdin C) *p*-vinylphenyl-3-nitroso-4-hydroxybenzoate functional group as the third group.

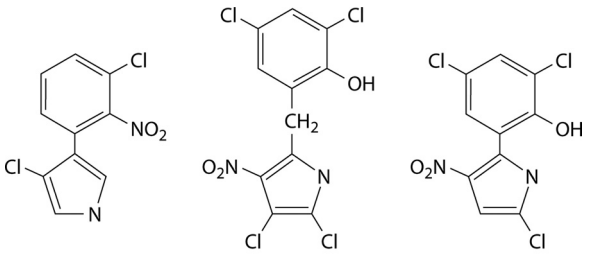


FIG. 6. Nitroaromatic phenylpyrrole antibiotics.

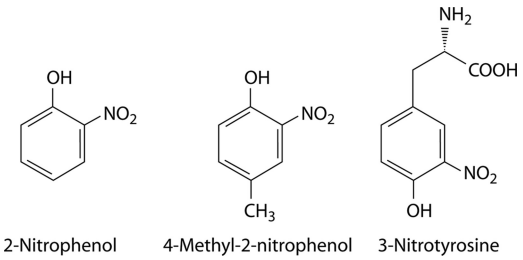


FIG. 8. Nitroaromatic signaling molecules.

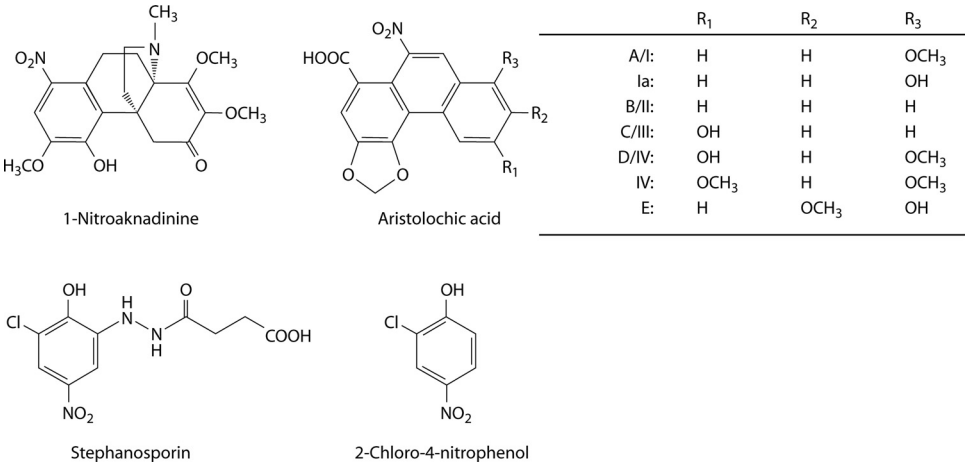
certain cardiovascular diseases. Viridomycin F had weak insecticidal and nematocidal activities (136). Viridomycin A, its free ligand 4-hydroxy-3-nitrosobenzaldehyde, and the ligand complexed with copper, nickel, iron, or cobalt displayed antibiotic activity against a range of different bacteria (212).

Several Gram-negative bacteria, including several *Pseudomonas* (2, 43, 115) and *Burkholderia* (44, 119, 164) strains, *Corallococcus exiguus*, *Cystobacter ferrugineus*, *Myxococcus fulvus* (50), and *Enterobacter agglomerans* (26), produce pyrrolnitrin (Fig. 6), a chloro-nitroarene metabolite with antifungal activity (2, 119). Pyrrolomycins A, B, and E (Fig. 6) are produced by the actinomycete *Actinosporangium vitaminophilum*. These compounds are active against not only fungi but also some Gram-negative and Gram-positive bacteria (45, 46).

Nitroaromatic compounds are also bioactive metabolites found in plants and fungi (Fig. 7). 1-Nitroaknadinine is an alkaloid from *Stephania sutchuenensis*, a traditional Chinese herbal plant used to alleviate arthritis and sore throats (114, 202). Chinese herbs of the genus *Aristolochia* were added to weight loss supplements but were abruptly discontinued when the bioactive compounds aristolochic acids I and II, two unusual nitrophenanthrene derivatives, were found to cause severe kidney damage (31). Investigations of the carrot truffle, *Stephanospora caroticolor*, revealed that the chloronitroarene stephanosporin and its breakdown product, 2-chloro-4-nitrophenol, are the compounds responsible for the bright orange pigmentation and may be produced as a chemical deterrent against predation (103).

Chemicals containing a nitro group are also found to be important in cellular signaling and in stimulating behavioral responses (Fig. 8). 2-Nitrophenol and 4-methyl-2-nitrophenol, which are present as rumen metabolites, are pheromones for ticks to aggregate and attach to mammals (36). Nitration of aromatic amino acids can occur in mammals, resulting in proteins with altered function (154). Although the biological significance of protein nitration remains unclear, 3-nitrotyrosine levels are elevated in patients with cardiovascular disease, suggesting that this molecule may be a useful indicator for certain types of physiological dysfunctions (175). Thaxtomins (Fig. 3) have been shown to be essential pathogenicity factors for the infection of plant tubers and for scab disease caused by *Streptomyces* strains (74, 116). The biosynthetic genes for thaxtomins are associated with transmissible DNA elements, which may explain the distribution and spread of this infectious phenotype among the members of this genus (12, 88, 96, 116).

Studies of these biogenic nitroaromatic compounds have revealed two methods for their synthesis. Similar to industrial organic syntheses, electrophilic attack by a nitronium cation can be used to directly attach the nitro group to an aromatic ring. Dioxyapyrrolomycin, 1-nitroaknadinine, 3-nitrotyrosine, and thaxtomins appear to be produced using this mechanism. In the biosynthesis of these compounds, formation of the nitronium cation was linked to the production of nitric oxide



	R_1	R_2	R_3
A/I:	H	H	OCH_3
Ia:	H	H	OH
B/II:	H	H	H
C/III:	OH	H	H
D/IV:	OH	H	OCH_3
IV:	OCH_3	H	OCH_3
E:	H	OCH_3	OH

FIG. 7. Nitroaromatic metabolites produced by plants and fungi.

radicals and, in some cases, to the activity of nitric oxide synthase (97, 116, 154, 205).

Alternatively, biosynthesis of nitroarenes can also proceed by direct oxidation of amino groups by specialized amine-oxygenases (N-oxygenases) (205). The best-characterized examples of N-oxygenases are from the biosynthesis of pyrrolnitrin and aureothin. The multicomponent oxygenase PrnD from *Pseudomonas fluorescens* catalyzes the conversion of aminopyrrolnitrin to pyrrolnitrin by using molecular oxygen as the substrate (63, 99, 104). Biochemical characterization confirmed bioinformatic predictions that PrnD is indeed a Rieske-type oxygenase that uses NAD(P)H and flavin as electron donors. Although in early studies the associated electron carrier proteins (reductase in two-component systems and reductase and ferredoxin in three-component systems) remained elusive, genome sequencing of *Pseudomonas fluorescens* Pf-5 allowed the successful identification of the flavin:NADH reductase PrnF. The *prnF* gene was found to be located approximately 1 kb downstream of the original four pyrrolnitrin biosynthesis genes, and activity assays with purified proteins showed that PrnF is indeed the true interacting partner for the aminopyrrolnitrin oxygenase PrnD (106). Molecular modeling of PrnD by use of the α-subunit of naphthalene dioxygenase (NahAc) from *Pseudomonas* sp. strain NCIB 9816-4 was surprisingly successful in identifying the amino acids lining the substrate binding pocket, despite only 19% sequence identity between the two enzymes. Mutagenesis of the predicted active site residues resulted in variants with altered substrate specificities or improved catalytic efficiencies (105).

In *Streptomyces thioluteus*, the amine-oxygenase AurF catalyzes the oxidation of *p*-aminobenzoate to *p*-nitrobenzoate for the biosynthesis of aureothin. By measuring whole-cell activities of *Streptomyces lividans* containing genes for wild-type and deletion variants of the aureothin pathway on expression plasmids, AurF was proposed to sequentially oxidize *p*-aminobenzoate to *p*-nitrobenzoate (66, 206). AurF was independently purified and characterized, showing that *p*-hydroxylaminobenzoate and *p*-nitrosobenzoate are intermediates of the reaction (28). Analysis of crystal structures, combined with previous biochemical spectroscopy studies, confirmed that AurF contains two iron atoms in the active site (28, 176). The only other characterized homologs of AurF are NorF from *Streptomyces orinoci* HKI-0260 and SpnF from *Streptomyces spectabilis*, which are responsible for the biosynthesis of orinocin in *S. orinoci* (195) and of neoaureothin (spectinabilin) in *S. spectabilis* (27), respectively. Both of these compounds differ from aureothin only in the lengths of their polyketide backbones, and their biosynthetic clusters are suggested to have evolved by gene duplication from a common ancestor (195).

In genetic studies of *Streptomyces venezuelae* ISP5230, a partial gene cluster for chloramphenicol biosynthesis was identified (67). However, the gene encoding the enzyme that catalyzes the final step in chloramphenicol biosynthesis, the oxidation of the amino group on *N*-dichloroacetyl-*p*-aminophenylserinol into a nitro group, was not clearly annotated. CmlI, encoded by a gene within the chloramphenicol cluster, was later proposed to be an N-oxygenase (139), but the enzyme has yet to be characterized functionally. Sequence comparisons show that it shares only 34% amino acid identity with AurF.

Haloperoxidases are able to catalyze the oxidation of amines into nitro groups, but only under artificial reaction conditions, such as at low pH, with excess hydrogen peroxide, and in the absence of their native substrates. Chloroperoxidase from the mold *Caldariomyces fumago* is able to oxidize 4-chloroaminobenzene into 4-chloronitrosobenzene at low pH (30, 172). Similarly, the bromoperoxidase from a strain of *Pseudomonas putida*, which naturally brominates aniline to *o*-, *m*-, and *p*-bromoanilines, is able to oxidize aniline into nitrobenzene when bromine is absent (80). However, it should be noted that both of these oxidoreductases catalyze N-oxidation in a fortuitous reaction and are not known to be part of any biosynthetic pathway for nitroarene metabolites.

ENVIRONMENTAL CONTAMINATION BY NITROAROMATIC COMPOUNDS

Nitroaromatic compounds are acutely toxic and mutagenic, and many are suspected or established carcinogens (117, 140, 152, 162). Several are listed on the U.S. Environmental Protection Agency's list of priority pollutants (95, 196a). The same properties that allow nitroaromatic compounds to be useful in chemical applications also make them hazardous to the health of both humans and wildlife. The interactions of nitroaromatic compounds with DNA and the resulting mutagenicity have been characterized extensively and reviewed for a variety of monocyclic, polycyclic, and heterocyclic nitroaromatic compounds (152). Through the use of the Ames *Salmonella* tester strains, *Escherichia coli* strains with defects in DNA repair, and mammalian cell lines, these compounds have been shown to cause transitions, transversions, and frameshift mutations in gene coding sequences (152). Oxidation and reduction products of nitroaromatic compounds can damage DNA directly or cause the formation of adducts that induce mutagenesis by misincorporation of nucleotides during DNA synthesis. Structural and spectroscopic studies have found that the position of the nitro group on the aromatic ring and the presence of other functional groups can influence the mutagenicity and carcinogenicity of these chemicals (152).

An unfortunate consequence of the widespread use of nitroaromatic compounds is environmental contamination of soil and groundwater. Although some nitroaromatic compounds are intentionally applied to the environment (i.e., pesticides), improper handling and/or storage practices by both producers and users have resulted in their accidental release in the environment in nations throughout the world. The annual tonnage of chemicals released reflects the shear scale of this problem. In 2002, approximately 5.1 metric tons of nitrobenzene and 1.1 metric tons of 2,4-dinitrotoluene were released into soil in the United States alone (204).

The manufacture, storage, and handling of munitions have left a legacy of environmental contamination by nitroaromatic compounds. As of June 2009, there were 70 Superfund sites throughout the United States (as defined by the 1980 Comprehensive Environmental Response, Compensation, and Liability Act [CERCLA]) that are contaminated with nitroarene explosives or their chemical precursors (197). Only 14 have been removed from the national priority list as having been completely remediated. In addition to these Superfund sites, the army ammunition plants that have produced explosives for the U.S. military are the most highly contaminated locations

(183). Environmental contamination from the production of explosives also exists in Germany, and detailed site characterizations and evaluation of remediation technologies are under way (183).

Industrial accidents have also resulted in environmental contamination by nitroaromatic compounds. The most prominent contamination event in recent history occurred on 13 November 2005, when a nitration unit (used in the first stage of aniline production) at a chemical manufacturing plant owned by China National Petroleum exploded in Jilin City, China (102). In addition to injuring over 70 people and claiming the lives of 6 workers, an estimated 100 tons of benzene and nitrobenzene flowed into the Songhua River (102). The resulting pollution contained benzene and nitrobenzene at concentrations several times above safe levels and shut down water plants in Jilin and also Harbin, a city downstream of the initial accident site with a population of approximately 10 million (213). Within a month, the toxic chemical slick from the Songhua River merged into the Amur River, flowing through Russia, before entering the Straits of Tartary and the Pacific Ocean (6).

Nitro-PAHs formed from atmospheric radical chemistry of PAHs contribute to air pollution in urban settings. Although the exhaust from all combustion engines contains hydrocarbons that are subject to nitration, the greatest source of atmospheric pollution is diesel engine-powered motor vehicles (165). Diesel exhaust contains PAHs such as naphthalene, acenaphthene, fluorene, anthracene, and pyrene, which themselves have mutagenic and carcinogenic properties and are on the EPA's list of priority pollutants (95). Addition of the nitro group further increases the toxicity of these compounds and their threat to human health (165).

BACTERIAL DEGRADATION OF NITROAROMATIC COMPOUNDS

Remarkably, bacteria have been isolated that are able to use several industrial nitroaromatic compounds, including nitrobenzene, nitrobenzoates, nitrophenols, nitrotoluenes, and chloronitrobenzenes, as carbon, nitrogen, and energy sources for growth. Detailed studies have revealed that the general strategy used to metabolize nitroaromatic compounds is analogous to the oxidative pathways for aromatic acid and aromatic hydrocarbon metabolism, but with appropriate modifications to accommodate the nitro group. Initial substrates are first converted to substituted phenols, quinones, or catechols, which are then metabolized to intermediates of the tricarboxylic acid (TCA) cycle. In some cases, reduction of the nitro group precedes oxidation of the aromatic ring.

Pathways for Nitrobenzoate and Nitrobenzaldehyde Catabolism

Several strains of *Nocardia* were isolated by Cain et al. in the late 1950s for their ability to use 2-, 3-, or 4-nitrobenzoate as the sole carbon, nitrogen, and energy source for growth, but the degradation pathways were not fully characterized (16, 20). Analysis of more recently isolated strains has led to the identification of genes and enzymes for nitrobenzoate metabolism. In *Pseudomonas fluorescens* KU-7 (65), a NADH:FMN reductase (NbaA) reduces 2-nitrobenzoate to 2-hydroxylaminobenzoate, which is then transformed by a mutase (NbaB) to 3-hydroxyanthranilate (Fig. 9) (81). Ring cleavage and decarboxylation are catalyzed by 3-hydroxyanthranilate 3,4-dioxygenase (NbaC) and 2-amino-3-carboxymuconate-6-semialdehyde decarboxylase (NbaD), producing 2-aminomuconic semialdehyde (123). Metabolism is completed by the formation of pyruvate and acetaldehyde, using a pathway like that of *Pseudomonas* sp. AP3 (123). In *Ralstonia* sp. strain SJ98 (Fig. 9), 2-nitrobenzoate is apparently metabolized by a reductive route, as ammonia was released and 2-aminobenzoate (anthranilate) was detected as an intermediate by gas chromatography-mass spectrometry (166). However, growth on anthranilate was not tested, and the genes and enzymes for 2-nitrobenzoate catabolism in strain SJ98 have yet to be identified. Interestingly, *Arthrobacter protophormiae* RKJ100 appears to contain both reductive pathways, but details about the enzymes involved have not been reported (24, 142).

Comamonas sp. strain JS46 and *Pseudomonas* sp. strain JS51 grow on 3-nitrobenzoate by use of an oxidative pathway (Fig. 9). Through the use of $^{18}O_2$ incorporation experiments, the oxidation of 3-nitrobenzoate to protocatechuate was shown to be catalyzed by a dioxygenase in both JS46 and JS51 (126). The genes encoding 3-nitrobenzoate dioxygenase in JS46 were later localized to a region of the chromosome that is flanked on both sides by IS*1071* elements, which may explain why the degradation phenotype of this strain is unstable (151).

Comamonas acidovorans NBA-10 (56), *Ralstonia pickettii* YH105 (211), *Ralstonia* sp. SJ98 (166), *Pseudomonas* sp. strain 4NT (60), and *Pseudomonas putida* TW3 (83, 161) all use a reductive pathway that results in protocatechuate as the key intermediate in the catabolism of 4-nitrobenzoate (Fig. 9). Among these strains, TW3 is the most extensively characterized. The genes encoding enzymes for the entire 4-nitrobenzoate degradation pathway have been identified, and key steps have been analyzed biochemically. In *P. putida* TW3, reduction of 4-nitrobenzoate to 4-hydroxylaminobenzoate is catalyzed in a NAD(P)H-dependent reaction by 4-nitrobenzoate reductase (PnbA) (79). Subsequent deamination by 4-hydroxylaminobenzoate lyase (PnbB) produces ammonium and protocatechuate (79), which is metabolized using the β-ketoadipate pathway (Fig. 9) (84, 161). The mechanism of the corresponding 4-hydroxylaminobenzoate lyase from *Pseudomonas* sp. strain 4NT was proposed to involve an intramolecular rearrangement to form an imine intermediate, followed by a hydrolytic deamination to form protocatechuate (125).

The benzoate derivative 5-nitroanthranilate, which is produced biologically for an unknown purpose by *Streptomyces scabies* and is also produced industrially for the synthesis of nitroaromatic products and dyes, has been shown to serve as a sole carbon, nitrogen, and energy source for *Bradyrhizobium* sp. strain JS329 (153). 5-Nitroanthranilate is deaminated to form 5-nitrosalicylate, which is subject to *ortho* ring cleavage by an enzyme similar to salicylate 1,2-dioxygenase (153).

Although a degradation pathway has not yet been determined, *Pseudomonas* strains that grow on *o*-nitrobenzaldehyde were obtained from activated sludge samples from a municipal wastewater treatment plant in China. The characterized strain (*Pseudomonas* sp. ONBA-17) utilized *o*-nitrobenzaldehyde as a sole source of carbon and nitrogen (214).

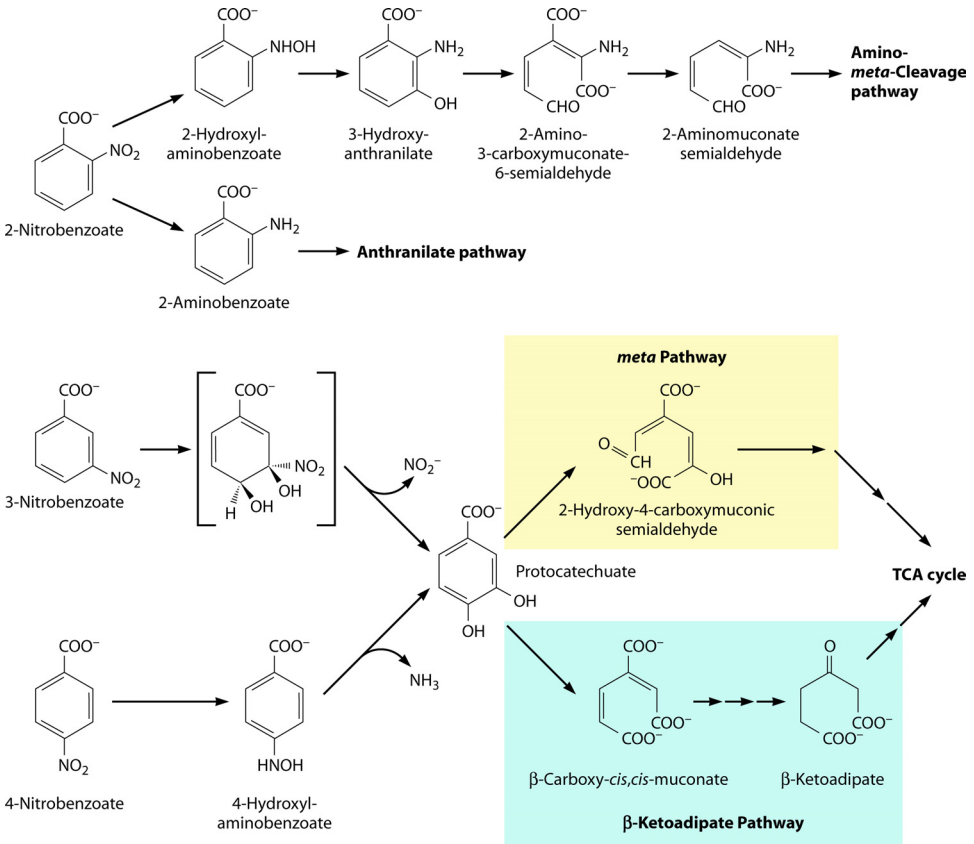


FIG. 9. Nitrobenzoate degradation pathways.

Pathways for Nitrophenol Catabolism

Several strains have been isolated by their ability to use mononitrophenols as sole carbon and energy sources for growth. *Pseudomonas putida* B2 grows on both 2- and 3-nitrophenol, but by use of different pathways (215). 2-Nitrophenol is oxidized by a NADPH-dependent monooxygenase through *o*-benzoquinone to produce nitrite and catechol (216), which is further metabolized by the β-ketoadipate pathway (215, 217) (Fig. 9 and 10). In contrast, 3-nitrophenol is reduced to 3-hydroxylaminophenol by a nitroreductase and is then deaminated and rearranged by a lyase to 1,2,4-trihydroxybenzene (121). The genes encoding the enzymes for the oxidation of 2-nitrophenol degradation were only recently identified. Molecular characterization of *Alcaligenes* sp. strain NyZ215 revealed that the genes encoding the 2-nitrophenol monooxygenase (OnpA), 2-benzoquinone reductase (OnpB), and catechol 1,2-dioxygenase (OnpC) are transcribed as a single operon (210).

Two pathways are known for the metabolism of 3-nitrophenol, both starting with reduction to 3-hydroxylaminophenol by a NAD(P)H-dependent reductase (Fig. 10). In *P. putida* B2, metabolism proceeds by deamination and rearrangement of 3-hydroxylaminophenol to form 1,2,4-trihydroxybenzene (121, 215). In contrast, *Cupriavidus necator* (formerly *Ralstonia eutropha*) JMP134 rearranges 3-hydroxylaminophenol to aminohydroquinone, and ammonium is removed in the ring cleavage pathway (168). The same enzymes that are used by JMP134 for 3-nitrophenol metabolism also allow the strain to grow on 2-chloro-5-nitrophenol (169). Reduction of 2-chloro-5-nitrophenol produces 2-chloro-5-hydroxylaminophenol, which is then rearranged into 2-chloro-5-aminohydroquinone and then dechlorinated to aminohydroquinone (Fig. 10). While genome sequencing of JMP134 has led to the identification of possible genes encoding the enzymes of the 3-nitrophenol degradation pathway, they have yet to be verified experimentally (150).

Numerous strains have been isolated by growth on 4-nitrophenol. In *Arthrobacter* sp. strain JS443 (82), *Arthrobacter protophormiae* RKJ100 (23), *Bacillus sphaericus* JS905 (92), *Burkholderia cepacia* RKJ200 (25), *Ralstonia* sp. strain SJ98 (166), *Rhodococcus opacus* AS2 (167), *Rhodococcus erythropolis* AS3 (167), and *Serratia* sp. strain DS001 (141), 4-nitrophenol is oxidized to 4-nitrocatechol and then 1,2,4-trihydroxybenzene before ring cleavage (Fig. 10). Biochemical characterization of *B. sphaericus* JS905 demonstrated that an oxygenase and flavoprotein reductase are responsible for the initial two oxidations of 4-nitrophenol to 2-hydroxy-1,4-quinone (not shown) with the release of nitrite (92). In contrast, *Arthrobacter aurescens* TW17 (64), *Pseudomonas putida* JS444 (129), *Pseudomonas* sp. strain WBC-3 (218), a *Moraxella* sp. (182), *Rhodococcus opacus* SAO101 (100), and *Rhodococcus* sp. PN1 (192) all use a monooxygenase that directly oxidizes 4-nitrophenol to benzoquinone, followed by reduction to hydroquinone (182). The pathways converge at ring cleavage, where 1,2,4-trihydroxybenzene and hydroquinone are each converted into maleylacetate,

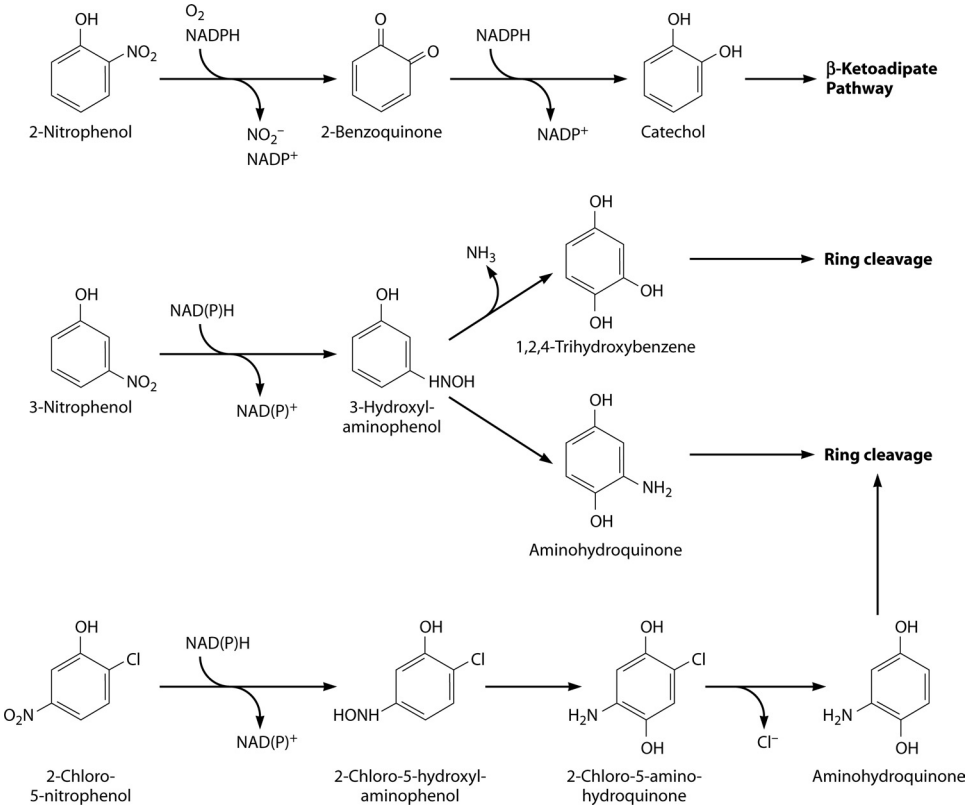


FIG. 10. Nitrophenol degradation pathways.

which is then reduced to β-ketoadipate and converted to TCA cycle intermediates. Interestingly, *Nocardia* sp. strain TW2 appears to contain both 1,2,4-trihydroxybenzene and hydroquinone pathways, which are differentially expressed in the presence of different chemical inducers (64). This may also be the situation in *Rhodococcus* strains PN1 and SAO101, although more detailed investigations will be required to understand the regulation of the two pathways in these strains. 4-Nitrophenol catabolism is part of the degradation pathway for the nitroaromatic pesticide methyl parathion in strains SD001 and WBC-3 and for 4-nitroanisole degradation in strains AS2 and AS3 (Fig. 11).

Only a few strains have been isolated that are able to grow on 2,4-dinitrophenol, 2,6-dinitrophenol, or 2,4,6-trinitrophenol (picric acid). The only strain that has been shown definitively to use 2,6-dinitrophenol as its sole carbon, nitrogen, and energy source for growth is *C. necator* JMP134 (41). The catabolic pathway for 2,6-dinitrophenol is quite different from the 3-nitrophenol pathway in JMP134 (Fig. 12A). A dioxygenase is used to oxidize 2,6-dinitrophenol to 4-nitropyrogallol with the release of the first nitro group. Ring cleavage of 4-nitropyrogallol produces 2-hydroxy-5-nitromuconate, which undergoes decarboxylation to 2-hydroxy-5-nitropenta-2,4-dienoic acid. Removal of the second nitro group is predicted to occur in the later steps of the pathway. The recently completed genome sequence of *C. necator* JMP134 (150) should aid in the identification of the genes and enzymes for the catabolism of 2,6-dinitrophenol.

Rhodococcus erythropolis strains HL24-1 and HL24-2 (110), *Rhodococcus* sp. strain RB1 (10), *Nocardioides* sp. strain CB22-2 (7), *Nocardioides simplex* FJ2-1A (159), and *Rhodococcus* sp. strain NJUST16 (173) are all able to grow using picric acid (2,4,6-trinitrophenol) and 2,4-dinitrophenol as sole carbon and/or nitrogen and energy sources. The degradation pathway was elucidated mainly by Knackmuss and colleagues (39, 40, 109, 110, 163) (Fig. 12B). A hydride-Meisenheimer complex is created by reducing picric acid with a NADPH-dependent reductase containing cofactor F_{420}, resulting in the removal of nitrite and the production of 2,4-dinitrophenol. A second reduction and hydride-Meisenheimer complex eventually results in hydrolytic cleavage of 2,4-dinitrophenol to 4,6-dinitrohexanoate, which may be metabolized through β-oxidation, using enzymes specialized in removing the remaining nitro groups. Some of the genes and enzymes of this pathway have been identified and characterized with respect to their function and regulation (75, 78, 127, 201).

Pathways for Nitrobenzene Catabolism

Two different strategies have evolved for degradation of nitrobenzene (Fig. 13). *Pseudomonas pseudoalcaligenes* JS45 was isolated from contaminated soil and groundwater collected from Pascagoula, MS, by its ability to grow on nitrobenzene as a sole carbon, nitrogen, and energy source (133). The genes encoding the entire pathway for nitrobenzene degradation in JS45 have been identified, and several of the enzymes have been characterized in great detail. Nitrobenzene is reduced to hydroxylaminobenzene (Fig. 13A)

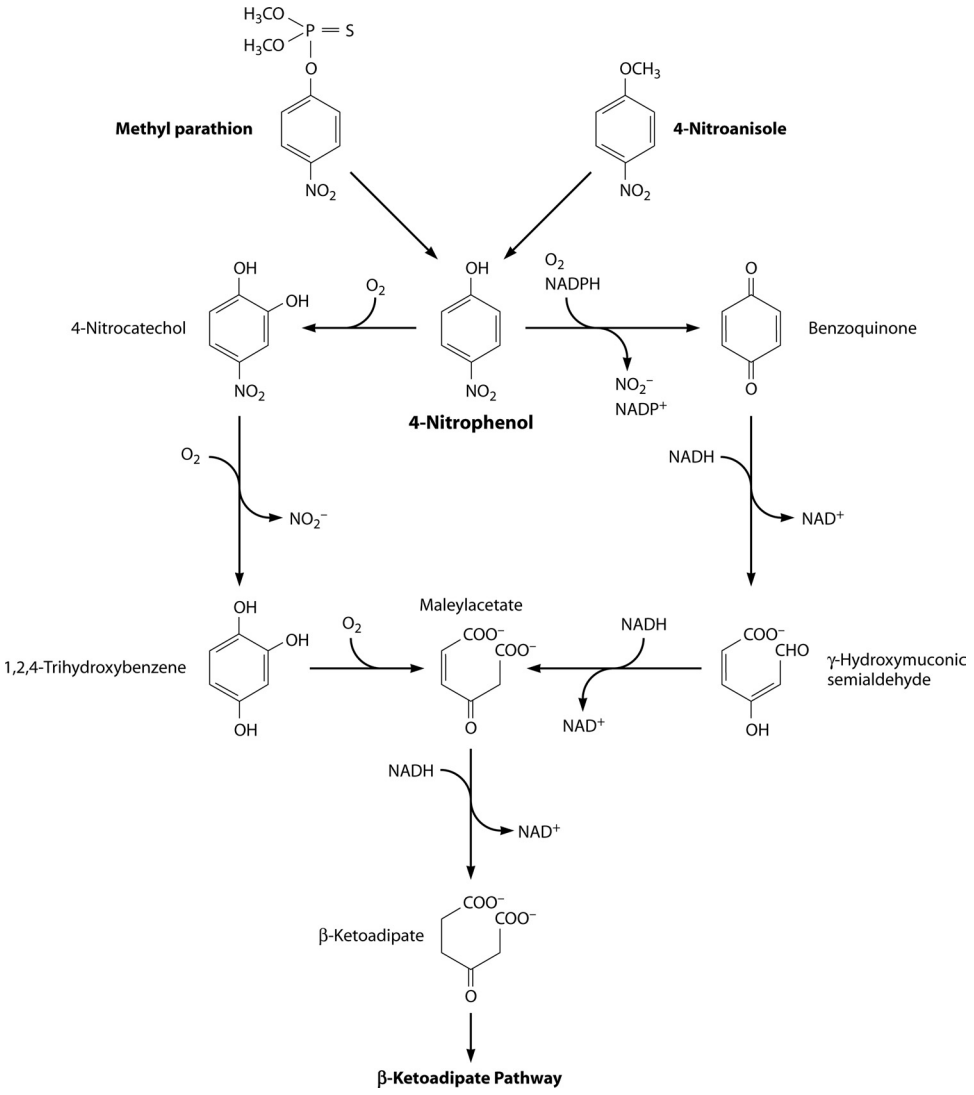


FIG. 11. Methyl parathion, 4-nitroanisole, and 4-nitrophenol degradation pathways.

through a nitrosobenzene intermediate (not shown) by the action of nitrobenzene nitroreductase (69, 133, 179). A mutase then isomerizes hydroxylaminobenzene to 2-aminophenol by intramolecular transfer of hydroxyl groups (34, 69, 125). 2-Aminophenol is further metabolized by a *meta*-cleavage pathway (124). Similar to catechol 2,3-dioxygenase, 2-aminophenol 1,6-dioxygenase breaks the benzene ring of 2-aminophenol to produce 2-aminomuconic semialdehyde (Fig. 13B). This product is subsequently oxidized in a NADH-dependent reaction to 2-aminomuconate, which is deaminated to form 4-oxalocrotonate (2-oxo-3-hexene-1,6-dioate). Metabolism then proceeds through decarboxylation, followed by hydrolysis and then cleavage by an aldolase, to eventually yield pyruvate and acetaldehyde (68, 71, 73). An acetaldehyde dehydrogenase scavenges the acetaldehyde by oxidation to acetate, which feeds into the TCA cycle.

Since the isolation of JS45, several other bacteria have been cultured that are also able to grow on nitrobenzene. *Pseudomonas* sp. strain AP-3 (191) and *Pseudomonas* sp. strain HS12 (147, 148) use similar pathways and enzymes for nitrobenzene degradation to those of JS45. However, in AP-3, 2-aminomuconate may undergo decarboxylation prior to deamination during the formation of 2-oxo-4-pentenoate (190, 191). *Streptomyces* sp. strain Z2 was isolated from a nitrobenzene-contaminated site in Dalian, China, and may also use the reductive pathway for growth on nitrobenzene, given its ability to grow on 2-aminophenol and picolinic acid (220). *Comamonas* sp. strain CNB-1 uses a pathway similar to that in JS45 for growth on both nitrobenzene and 4-chloronitrobenzene (209).

Considering that reduction of the nitro group is a highly favorable reaction [as the electronegativity of the N atom (+III) becomes satisfied by electrons donated from NAD(P)H], it is not surprising that the reductive pathway is prevalent in strains that have been isolated by growth on nitrobenzene. The lone exception is *Comamonas* sp. strain JS765, which grows on nitrobenzene by use of an oxidative

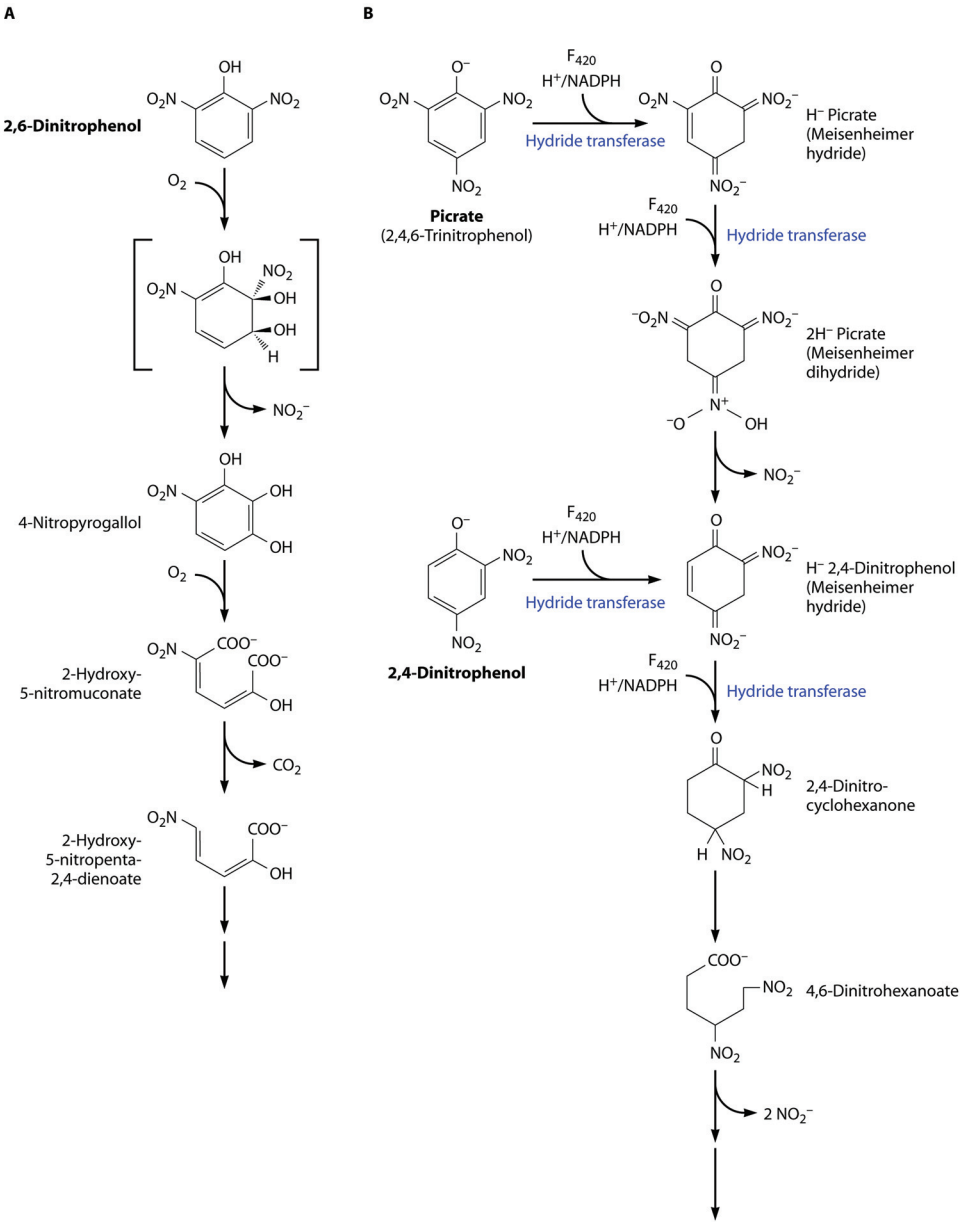


FIG. 12. Di- and trinitrophenol degradation pathways. (A) 2,6-Dinitrophenol degradation pathway; (B) 2,4-dinitrophenol and picric acid (2,4,6-trinitrophenol) degradation pathways.

pathway (134). Instead of using three steps to convert nitrobenzene to a substrate for ring cleavage, JS765 uses a dioxygenase to oxidize nitrobenzene to catechol in a single enzymatic step that results in the release of nitrite (Fig. 13C). Biochemical and genetic analyses of nitrobenzene dioxygenase (NBDO) showed that it belongs to the naphthalene family of multicomponent Rieske-type dioxygenases (111). Crystal structures and site-directed mutagenesis studies of NBDO identified the active site of the enzyme, as well as key amino acids that bind and direct oxidation specifically to the nitro-substituted carbon (48, 91). JS765 uses a standard *meta*-cleavage pathway (70, 134), like that in *P. putida* mt-2 (124) and other *Pseudomonas* strains (32), to metabolize catechol to acetaldehyde and pyruvate (Fig. 13D).

Pathways for Nitrotoluene Catabolism

Despite its isolation over 15 years ago, *Acidovorax* sp. strain JS42 still remains the only reported bacterium that is able to use 2-nitrotoluene as a sole carbon, nitrogen, and energy source for growth (62). In JS42, a dioxygenase oxidizes the 2 and 3 positions of 2-nitrotoluene to form an unstable nitrohydrodiol, which spontaneously rearranges to 3-methylcatechol with the release of nitrite (Fig. 14). A standard *meta*-cleavage pathway (Fig. 13D) is then used to complete metabolism of 3-methylcatechol to TCA cycle intermediates (62). Subsequent growth assays showed that JS42 is also able to grow on nitrobenzene (111). Cloning and sequencing of the genes encoding 2-nitrotoluene dioxygenase (2NTDO) revealed

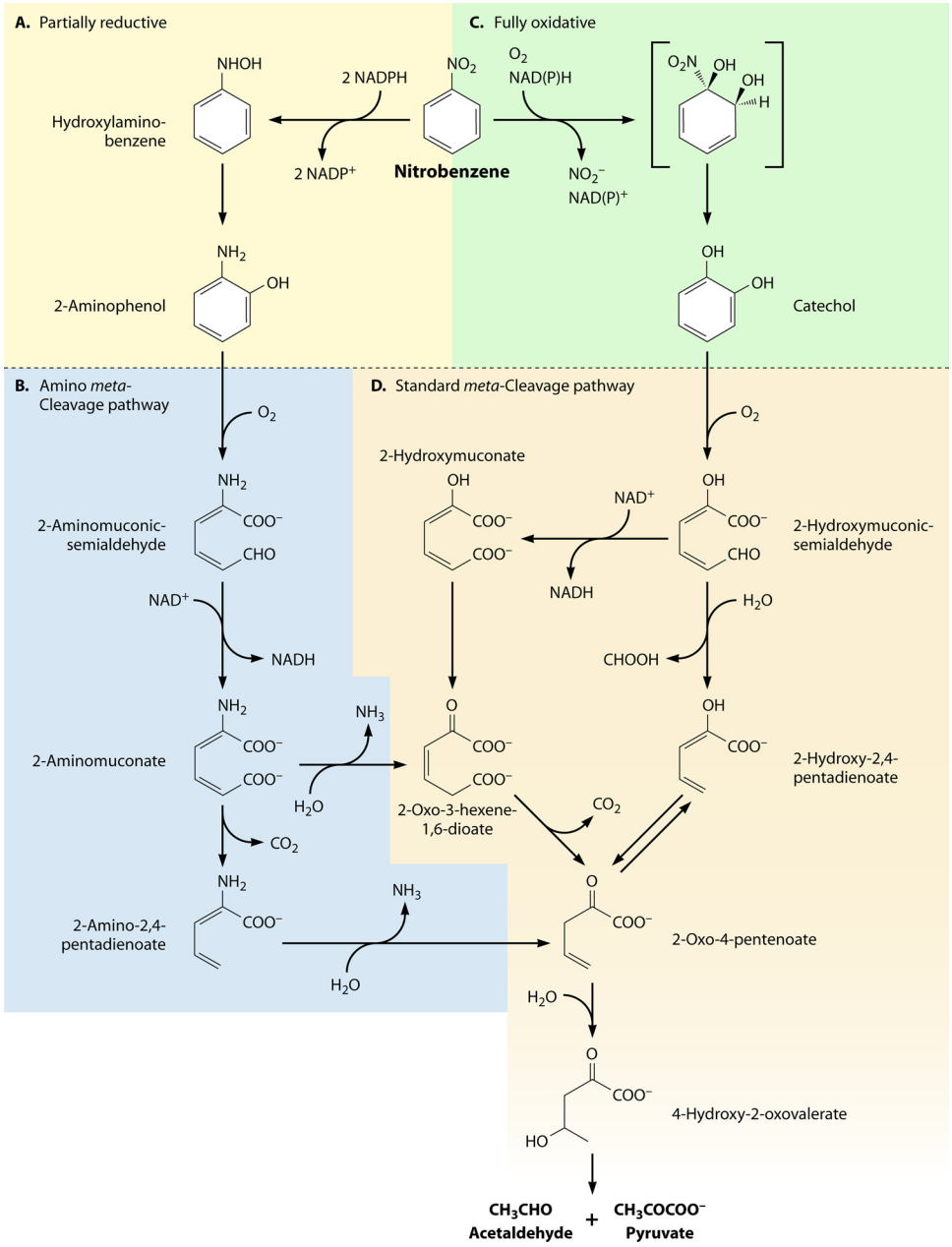


FIG. 13. Nitrobenzene degradation pathways.

that it is a Rieske-type nonheme iron dioxygenase (later verified by biochemical analysis of the purified protein [146]) which has high sequence similarity to naphthalene dioxygenase from *Pseudomonas* sp. strain 9816-4 (143). Mutagenesis studies revealed that specificity is controlled by the C-terminal half of the dioxygenase (144), and like the case in NBDO (91), the asparagine at position 258 is critical for proper positioning of substrates in the active site for oxidative removal of the nitro group (107). A divergently transcribed LysR-type regulator activates transcription of the 2NTDO genes in response to nitroaromatic compounds (89, 112).

In addition to growing on nitrobenzene, *Comamonas* sp. strain JS765 is able to use 3-nitrotoluene as the sole carbon, nitrogen, and energy source for growth (111). NBDO oxidizes 3-nitrotoluene to 4-methylcatechol (Fig. 14), which is cleaved by the same *meta*-cleavage pathway as that used for catechol in this strain (Fig. 13D). Aside from JS765, there have been no other reports of strains that are able to grow on 3-nitrotoluene. Using respirometry, enzyme assays, and chemical analysis of the degradation intermediates, *Pseudomonas putida* OU3 was shown to transform 3-nitrotoluene in stepwise reactions into 3-nitrobenzyl alcohol, 3-nitrobenzaldehyde, 3-nitrobenzoate, and finally, 3-nitrophenol (1). The nitro group is removed in subsequent transformations of 3-nitrophenol. However, strain OU3 was not shown to grow directly on 3-nitrotoluene or any of the degradation intermediates.

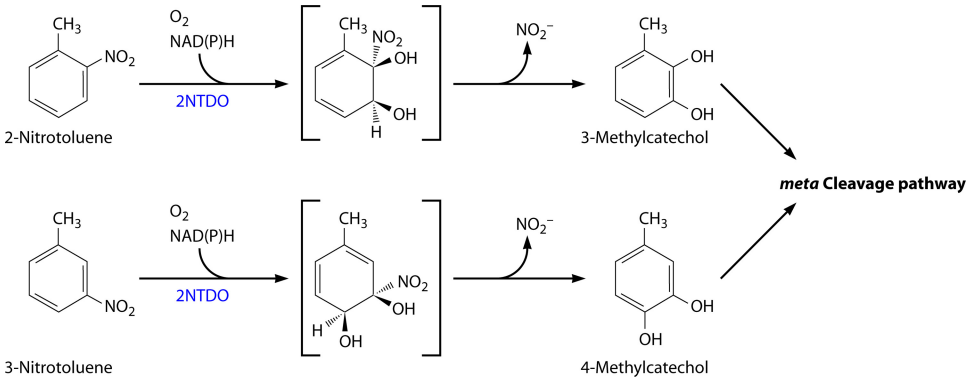


FIG. 14. Pathways for 2-nitrotoluene and 3-nitrotoluene degradation.

Three strains have been isolated by their growth on 4-nitrotoluene (4NT). *Mycobacterium* sp. strain HL 4-NT-1 initiates degradation of 4NT by reducing the nitro group to form 4-hydroxylaminotoluene, which is then converted to 6-amino-*m*-cresol (185) (Fig. 15). Similar to the case in the nitrobenzene degradation pathway in *P. pseudoalcaligenes* JS45 (Fig. 13A and B), the amino group is removed after *meta* ring cleavage (72). In contrast, degradation of 4NT in *Pseudomonas* sp. strain 4NT and *P. putida* TW3 is initiated by sequential oxidations at the methyl group to form 4-nitrobenzoate (Fig. 15). Following reduction to 4-hydroxylaminobenzoate, deamination occurs, resulting in the formation of protocatechuate. Depending on the strain, protocatechuate either enters the β-ketoadipate pathway, as in *P. putida* TW3 (161), or undergoes *meta* ring cleavage (*Pseudomonas* sp. 4NT [60]).

The degradation pathways for 2,4-dinitrotoluene and 2,6-dinitrotoluene are similar to those for nitrobenzene and 2-nitrotoluene in *Comamonas* sp. JS765 and *Acidovorax* sp. JS42 in that a Rieske-type dioxygenase catalyzes the initial oxidation and removal of a nitro group (Fig. 16). The products of this reaction are methylnitrocatechols, which are further degraded by slightly different pathways. In *Burkholderia* sp. strain DNT (58, 59, 61, 184, 186, 187) and *Burkholderia cepacia* R34 (85, 86, 131), the 4-methyl-5-nitrocatechol produced from 2,4-dinitrotoluene is oxidized by a monooxygenase to remove the second nitro group, forming 2,4,5-trihydroxytoluene, which is a substrate for *meta* ring cleavage (85). In contrast, metabolism of 2,6-dinitrotoluene by *B. cepacia* JS850 and *Hydrogenophaga palleronii* JS863 yields 3-methyl-4-nitrocatechol (Fig. 16), which is a direct substrate for *meta* ring cleavage; trihydroxytoluene does not appear to be an intermediate as in 2,4-dinitrotoluene degradation, and the second nitro group is removed after ring cleavage (131).

To date, no strains that grow using TNT as a sole carbon and energy source have been isolated successfully. Although Ramos et al. described *Pseudomonas* sp. clone A as being able to use TNT as the sole nitrogen source for growth by formation of a hydride-Meisenheimer complex (37, 57), detailed chemical analysis and identification of the TNT transformation intermediates by Knackmuss et al. for this and other strains disproved these claims (200). Two Gram-positive isolates, strains TNT-8 and TNT-32, were able to use TNT as a nitrogen source, but the mechanism of nitrogen assimilation remains unclear (200). More recently, Ramos et al. reported that *Pseudomonas putida* JLR11 (13–15) and *Escherichia coli* AB1157 (51) were able to use TNT as a nitrogen source for growth, reducing the nitro group and recovering the ammonium by the use of nitroreductases. The nitroreductases NfsA and NfsB, together with the *N*-ethylmaleimide reductase NemA, contributed to the ability of *E. coli* AB1157 to obtain usable nitrogen from TNT (51). Similarly, the nitroreductase PrnA was shown to be involved in the utilization of TNT as a nitrogen source by *P. putida* JLR11, and the assimilatory nitrite reductase NasB contributed to the ability of the strain to grow efficiently (14, 15). A mechanism for the release of nitrite during the condensation of hydroxylaminodinitrotoluene (the product of nitroreductase activity on TNT) and a Meisenheimer dihydride complex (Fig. 12B) of TNT to form a diarylamine was proposed based on studies with ^{15}N-labeled TNT (207).

Pathways for Chloronitrobenzene Catabolism

Only four strains have been described that can use chloronitrobenzenes as sole carbon and energy sources for growth. *Pseudomonas stutzeri* ZWLR2-1 was isolated by its ability to grow on 2-chloronitrobenzene, and it was reported to release chloride and nitrite from this substrate (113). However, further characterization of its degradation pathway has not been reported. *Comamonas* sp. strain CNB-1 (209), *Pseudomonas putida* ZWL73 (219), and *Comamonas* sp. strain LW1 (94) each use a nitroreductase to reduce 4-chloronitrobenzene to 1-chloro-4-hydroxylaminobenzene, which is further transformed to 2-amino-5-chlorophenol by a hydroxylaminobenzene mutase or via Bamberger rearrangement (Fig. 17A). Ring cleavage by 2-aminophenol 1,6-dioxygenase produces 2-amino-5-chloromuconate, which is converted to TCA cycle intermediates after additional enzymatic steps (209). Recently, mutant forms of nitrobenzene dioxygenase from *Comamonas* sp. JS765 (91) were used to engineer the chlorobenzene-degrading strain *Ralstonia* sp. JS705 to grow on all three isomers of chloronitrobenzene (Fig. 17B) (90).

Pathways for Catabolism of Biologically Produced Nitroaromatic Compounds

Very little is understood about how biologically synthesized nitroaromatic compounds are degraded in the natural environ-

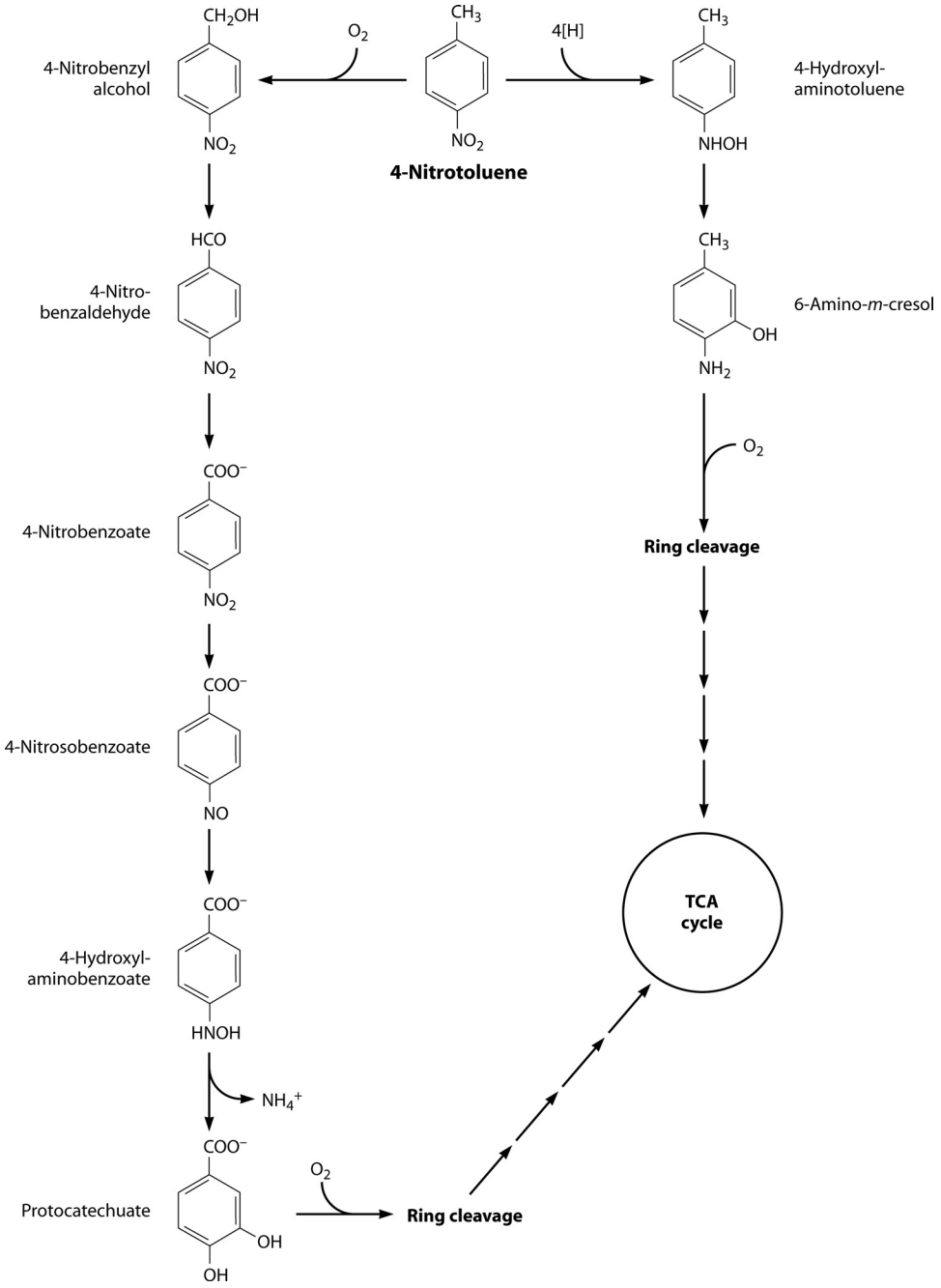


FIG. 15. 4-Nitrotoluene degradation pathways.

ment. Despite the widespread use of chloramphenicol in hospitals and research laboratories throughout the world for over 50 years, microbial pathways for its degradation are not yet understood. There is growing interest in understanding the metabolic fate of naturally occurring nitroaromatic compounds, but besides 5-nitroanthranilate (see above) (153), 3-nitrotyrosine is currently the only biogenic nitroaromatic compound on which bacterial strains have been reported to grow. Isolated from soil collected from Cape Cod, MA, *Burkholderia* sp. strain JS165 and *Variovorax paradoxus* JS171 are able to use 3-nitrotyrosine as the sole carbon, nitrogen, and energy source for growth (130). 3-Nitrotyrosine is converted to 4-hydroxy-3-nitro-phenylacetate by use of an α-ketoglutarate-dependent deaminase (Fig. 18). A NADH-dependent denitratase then removes the nitro group to produce homoprotocatechuate, which is metabolized by a tyrosine salvage pathway. The gene encoding the denitratase as been identified, and characterization of the purified protein showed that it is a previously uncharacterized flavoprotein monooxygenase which appears to be widely distributed in several genera of bacteria (149).

In rat cells, 3-nitrotyrosine is converted to 4-hydroxy-3-nitrophenylacetate through the formation of 3-nitrotyramine and

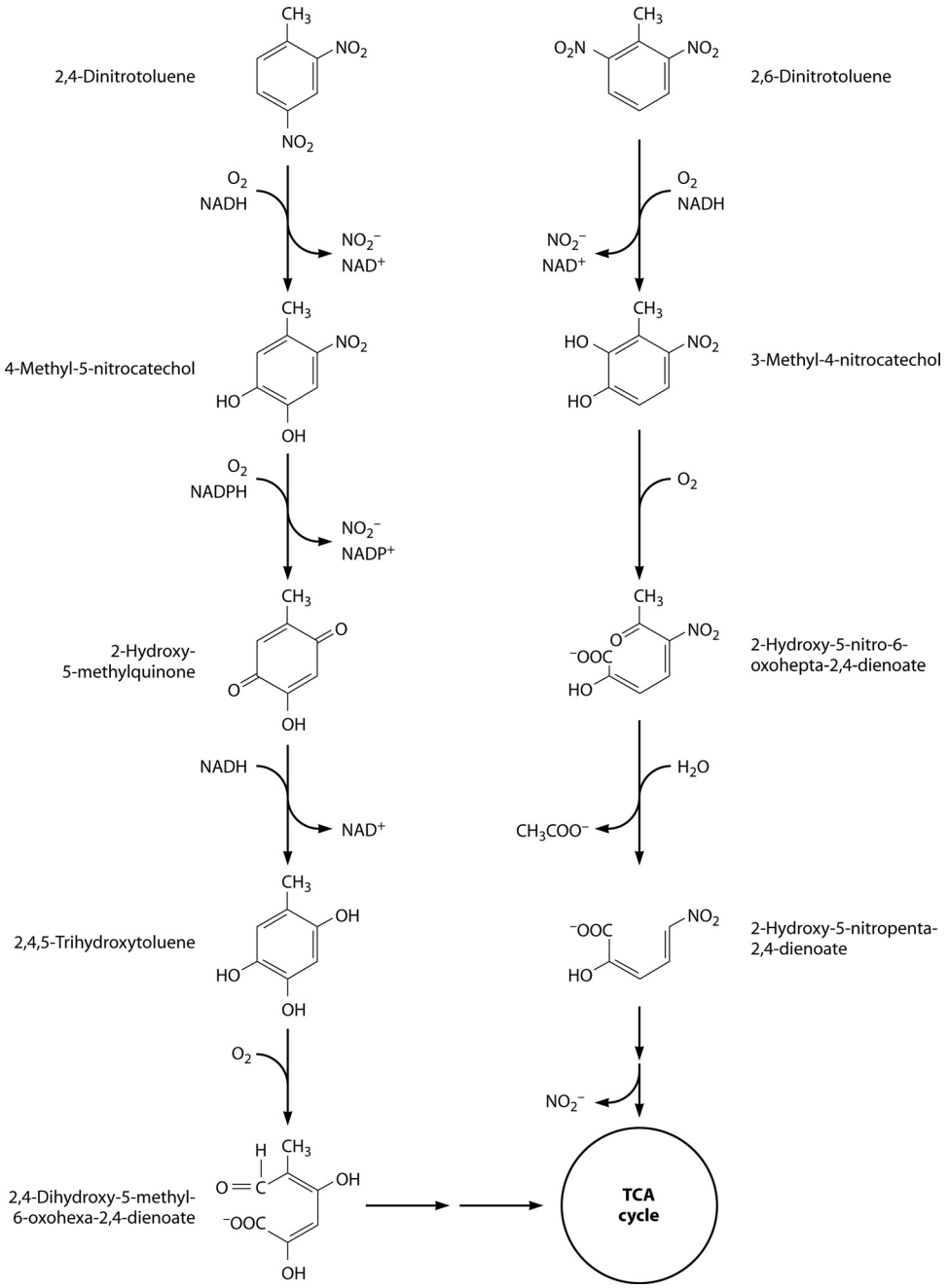


FIG. 16. Degradation pathways for 2,4-dinitrotoluene and 2,6-dinitrotoluene.

4-hydroxy-3-nitrophenylacetaldehyde intermediates (9). *Escherichia coli* MG1655 can use 3-nitrotyramine as a sole nitrogen source for growth but cannot use 3-nitrotyrosine (160). Similar to mammalian cells, MG1655 uses an amine oxidase (TynA) to remove the terminal amino group from 3-nitrotyramine to produce 4-hydroxy-3-nitrophenylacetaldehyde (Fig. 18), which is then oxidized by phenylacetaldehyde dehydrogenase (FeaB). 4-Hydroxy-3-phenylacetate is a dead-end metabolite in MG1655, as the strain appears to lack the enzymes present in *Burkholderia* sp. strain JS165 and *Variovorax paradoxus* JS171 that complete metabolism to compounds that enter the TCA cycle. Interestingly, expression of both *tynA* and *feaB* was under the regulatory control of the nitric oxide-sensitive repressor (NsrR), further supporting the link between nitric oxide production and the nitration of tyrosine residues in proteins (160).

EVOLUTIONARY ORIGINS OF THE OXIDATIVE PATHWAYS FOR NITROBENZENE AND NITROTOLUENE DEGRADATION

With the exception of 3-nitrotyrosine and 5-nitroanthranilate, the biodegradation pathways for all of the aforemen-

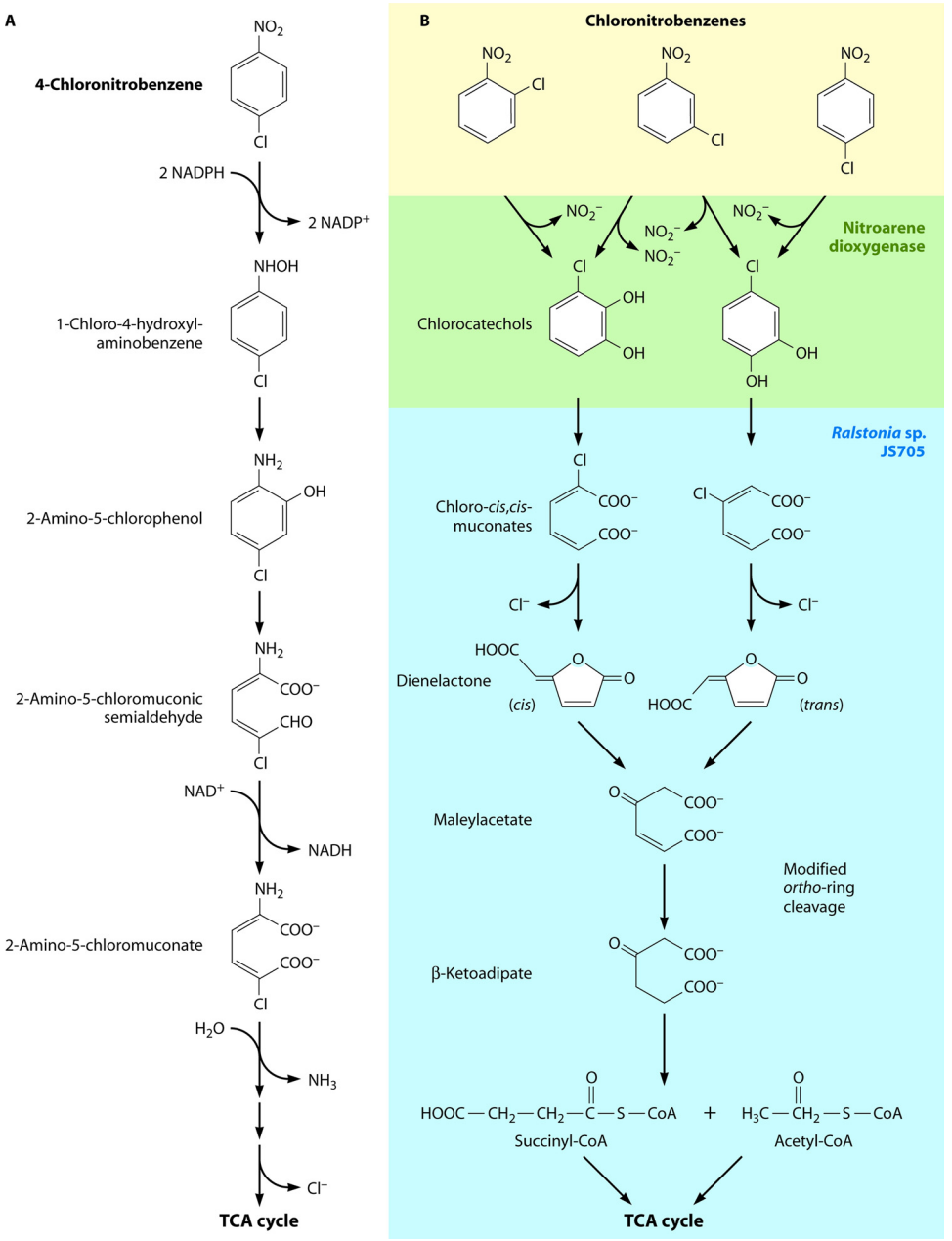


FIG. 17. Chloronitrobenzene degradation pathways. (A) Pathways found in natural isolates. (B) Engineered pathway in *Ralstonia* sp. JS705. (Panel B adapted from reference 90 with permission of Blackwell Publishing Ltd.)

tioned nitroaromatic compounds are for synthetic chemicals that are not biologically produced and have been present in the environment in significant quantity only since the industrial revolution. Natural selection has apparently driven the evolution of microorganisms that not only are able to tolerate these toxic contaminants but have adapted their metabolism to take advantage of these unique carbon, nitrogen, and energy sources for growth.

The most striking example of this rapid evolution is seen within strains that use oxidative pathways for nitrobenzene and nitrotoluene catabolism. It is clear from studies on the regulation and biochemistry of the nitroarene dioxygenase enzymes from *Comamonas* sp. strain JS765, *Acidovorax* sp. strain JS42, and *Burkholderia* sp. strains R34 and DNT that their pathways have evolutionary origins in a naphthalene degradation pathway like that present in *Ralstonia* sp. strain U2 (87, 111, 112, 186, 221). In all of these strains, the genes for the dioxygenase system are organized in very similar operons, and the deduced protein sequences share >85% identity (Fig. 19). In strain U2, naphthalene is initially oxidized to naphthalene *cis*-dihydrodiol and then converted to TCA cycle compounds, with salicylate and gentisate as key intermediates. Although the nitroarene dioxygenases from strains JS765, JS42, R34, and DNT are specialized in their ability to remove nitro groups from aro-

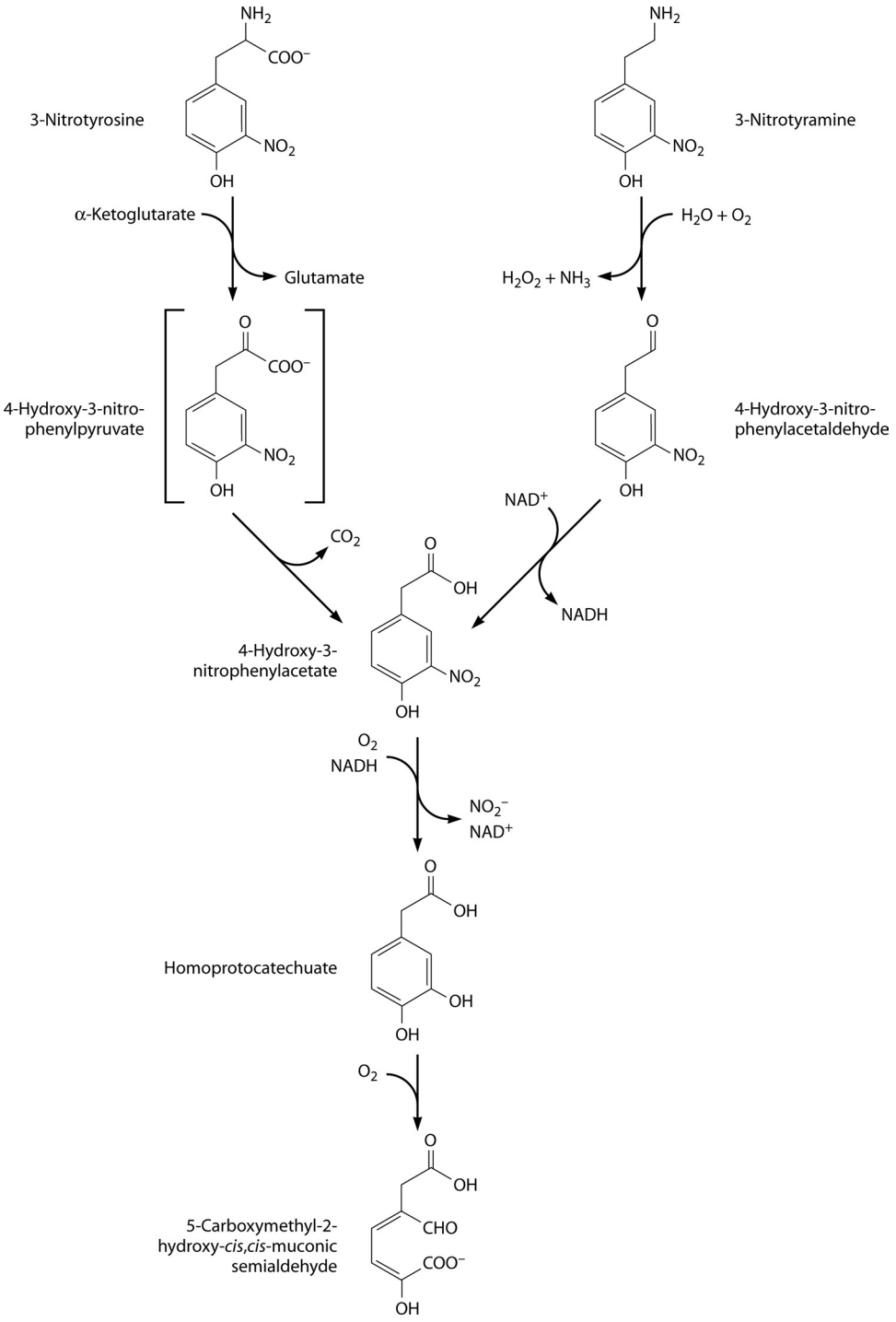
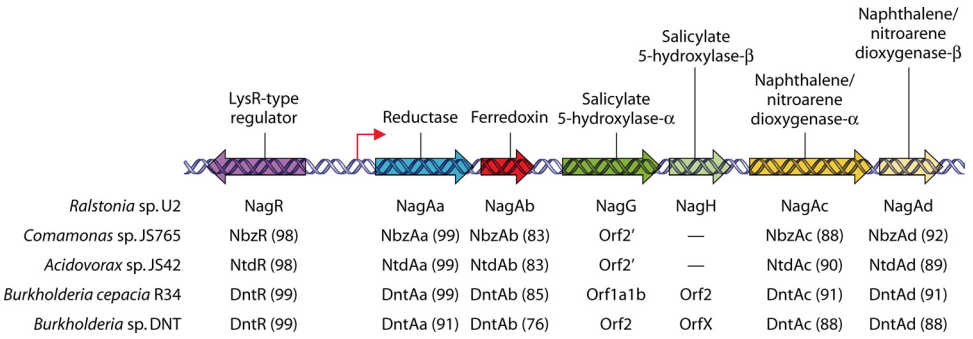


FIG. 18. Degradation pathways for 3-nitrotyrosine and 3-nitrotyramine.

matic rings, they still retain the ability to oxidize naphthalene to the *cis*-dihydrodiol (Fig. 20) (111, 144, 186). In contrast, naphthalene dioxygenases are unable to oxidize nitroarene substrates with concomitant removal of nitrite.

The presence of pseudogenes in the nitroarene dioxygenase gene clusters provides additional evidence for an ancestral relationship to naphthalene dioxygenase gene clusters. Remnants of the genes encoding a multicomponent salicylate 5-hydroxylase (*nagGH*; the enzyme oxidizes salicylate into gentisate) from the naphthalene degradation gene cluster are embedded in the nitroarene dioxygenase operons in these four strains (Fig. 19). In strains JS765 and JS42, *nagH* is completely absent and only the 5′ half of *nagG* remains; frameshift mutations and the absence of ribosome-binding sites preclude the production of functional proteins in *Burkholderia* strains R34 and DNT. Additionally, a gene similar to the gene encoding



Ralstonia sp. U2	NagR	NagAa	NagAb	NagG	NagH	NagAc	NagAd
Comamonas sp. JS765	NbzR (98)	NbzAa (99)	NbzAb (83)	Orf2′	—	NbzAc (88)	NbzAd (92)
Acidovorax sp. JS42	NtdR (98)	NtdAa (99)	NtdAb (83)	Orf2′	—	NtdAc (90)	NtdAd (89)
Burkholderia cepacia R34	DntR (99)	DntAa (99)	DntAb (85)	Orf1a1b	Orf2	DntAc (91)	DntAd (91)
Burkholderia sp. DNT	DntR (99)	DntAa (91)	DntAb (76)	Orf2	OrfX	DntAc (88)	DntAd (88)

FIG. 19. Dioxygenase gene clusters in naphthalene- and nitroarene-degrading bacteria. Numbers in parentheses denote amino acid identities shared with the corresponding protein components of naphthalene dioxygenase in *Ralstonia* sp. U2.

naphthalene *cis*-dihydrodiol dehydrogenase in *Ralstonia* sp. strain U2 is located downstream of the 2NTDO gene cluster in JS42, but it contains a frameshift mutation and its product is not functional (145). Neither of these enzymes is necessary for the degradation of nitroarene substrates, so it is not surprising that deletions and mutations resulting in their loss of function are present in the nitroarene-degrading strains.

This evolutionary link is further supported by investigations focusing on the regulation of these pathways. In *Comamonas* sp. strain JS765, *Acidovorax* sp. strain JS42, and *Ralstonia* sp. strain U2, the product of a divergently transcribed *lysR*-type regulatory gene located upstream of each dioxygenase operon (Fig. 19) activates gene expression in response to recognized inducer compounds (87, 89, 112). Sequence comparisons revealed that the regulators in strains JS42 and JS765 (NtdR and NbzR, which are identical in sequence) differ from the regulator in strain U2 (NagR) by only five amino acids. The LysR binding sites and promoters are identical in all three strains, and both regulators activate gene expression in the presence of salicylate, which is an intermediate of the naphthalene degradation pathway and the natural inducer of the naphthalene degradation genes in strain U2 (87, 112). However, neither naphthalene nor salicylate serves as a growth substrate for *Comamonas* sp. JS765 or *Acidovorax* sp. JS42 (111). Although the strains were isolated from geographically distinct locations, the nitroarene dioxygenase operons from strains JS765, JS42, R34, and DNT have many similar characteristics. The presence of transposable elements flanking these gene clusters suggests that horizontal gene transfer may have contributed to their distribution. Recent genome sequencing of JS42 revealed that the 2NTDO operon is flanked by an integrase gene (upstream) and an IS*4* transposase gene (downstream). Additionally, while the mean G+C content of the dioxygenase operon in JS42 is 57%, the overall average G+C content of the JS42 genome is significantly higher (66%).

CONCLUSIONS AND PERSPECTIVES

Much has been learned about the bacterial metabolism of nitroaromatic compounds, but several fundamental aspects regarding their biosynthesis and biodegradation have yet to be explored. Research in the last 5 decades on the biodegradation of nitroaromatic compounds has uncovered bacteria from contaminated environments that have evolved to use many of these chemicals as substrates for growth, and in-depth analyses of several of these strains have led to the identification and characterization of the genes and enzymes in their degradation pathways. Although we have gained many insights into the origins of nitroaromatic degradation pathways, several aspects of these pathways remain unknown. Some largely unexplored issues are the evolutionary history of the degradation pathways (i.e., the sum number of changes and the amount of time and order in which they occurred) and how they integrate into existing metabolic pathways and global regulatory control networks, such as catabolite repression (52) and nitrogen regulation (120). Even less well understood are the roles that the

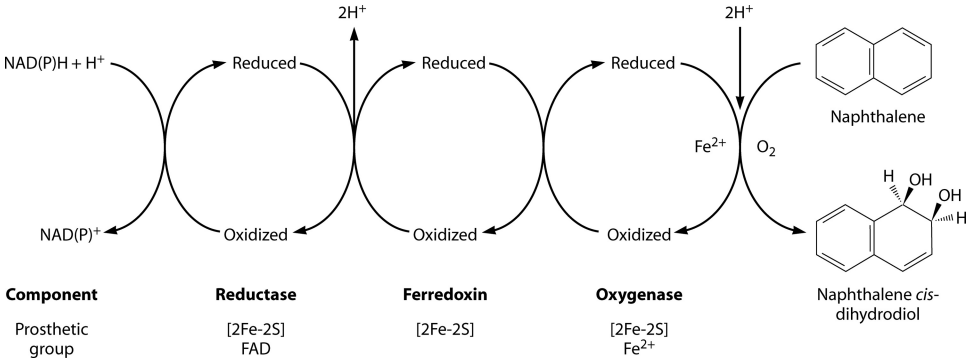


FIG. 20. The nitroarene dioxygenases have ancestral roots in the naphthalene dioxygenase enzyme system. Electrons are transferred from NAD(P)H through reductase and ferredoxin proteins to the catalytic (α) subunit of the dioxygenase to allow catalysis to occur.

local microbial ecology, chemical composition, and geophysical properties at contaminated environments may play in shaping the evolution of degradation pathways for nitroaromatic compounds. Investigations in these areas not only will shed light into adaptive pathway evolution in bacteria but also will provide valuable information that can be applied for biotreatment of environmental contamination by developing more effective methods for stimulating or accelerating natural attenuation and for engineering strains with improved biodegradation capabilities.

The total diversity of degradation pathways for synthetic nitroaromatic compounds remains unknown. The degradation of the more-complex nitroarenes, such as nitro-PAHs, has not been studied, although it seems that similar oxidation mechanisms are likely to be used for aerobic degradation. While transformation of nitroaromatic compounds in anoxic environments is well documented (183), their assimilation as carbon sources for growth by anaerobic bacteria remains an open field of study that has largely been unexplored. Given the importance of the nitro group in synthetic chemistry and the widespread application of nitroaromatic compounds in consumer and industrial products, the future may yield many new man-made nitroaromatic compounds and substrates for the evolution of degradation pathways.

Knowledge about the biosynthesis and biodegradation of biologically produced nitroaromatic compounds is also in its infancy. Although these compounds were initially isolated and characterized for their bioactive properties, their true biological roles and physiological significance to their hosts remain largely unknown. As more biogenic nitroaromatic compounds are discovered, it is likely that the enzymes involved in their biosynthesis may find use in the production of novel chemicals with a variety of applications. For example, nitrating enzymes may prove useful for modulating the activities of drug compounds by the addition of nitro groups. It is also possible that some of the biosynthesis intermediates will be pharmaceutically active or have antibiotic properties. Metabolism of nitroaromatic compounds in bacteria remains a rich field of study, and this and many other lines of investigation remain to be pursued in the future.

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