METABOLIC DIVERSITY INVOLVED IN BIODEGRADATION OF 2-NITROIMIDAZOLE AND 5-NITROANTHRANILIC ACID

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By

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METABOLIC DIVERSITY INVOLVED IN BIODEGRADATION OF 2-NITROIMIDAZOLE AND 5-NITROANTHRANILIC ACID

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To my wife and family

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF SYMBOLS AND ABBREVIATIONS

2NI	2-Nitroimidazole
4,6-DNH	4,6-Dnitrohexanoate
5NAA	5-Nitroanthranilic acid
5NSA	5-Nitrosalicylic acid
BCA	Bicinchoninic acid
BLK	Bruhn-Lenke-Knackmuss minimal media (without nitrogen)
Cm	Chloramphenicol
HAB	Hydroxylaminobenzene
HNPA	2-Hydroxy-5-nitropenta-2,4-dienoic acid
HPLC	High performance liquid chromatography
IM2O	Imidazol-2-one
IPTG	Isopropyl b-D-1-thiogalactopyranoside
LB	Luria-Bertani broth
MSA	Multiple sequence alignment
naaA	Gene encoding 5NAA deaminase (aminohydrolase)
naaB	Gene encoding 5NSA 1,2-dioxygenase
naaC	Gene encoding lactonase
NAO	Nitroalkane oxidase
NCBI	National Center for Biotechnology Information
NMO	Nitronate monooxygenase
nnh	Gene encoding 2-nitroimidazole nitrohydrolase
OD_{600}	Optical density, at 600 nanometers wavelength
ORF	Open reading frame
PCR	Polymerase chain reaction
RFP	Ring fission product
RT	Retention time
TSA	Tryptic soy agar
TSB	Tryptic soy broth

SUMMARY

Evolution of strategies for biodegradation of synthetic organic pollutants relies on recruitment of genes from catabolic pathways for natural compounds. Thus, investigation of metabolic diversity in nature can reveal biochemical strategies that could be recruited for bioremediation of pollutants. As part of a search for novel metabolic diversity, we isolated soil bacteria able to degrade 2-nitroimidazole (2NI) and 5-nitroanthranilic acid (5NAA) selected on the basis of unique structures, and determined the biochemistry and molecular biology of their biodegradation pathways.

2NI, produced by pseudomonads and *Actinomycetes*, is representative of many synthetic nitroimidazoles that are widely used in various fields from medicine to military applications, but nothing is known about their biodegradation mechanisms. *Mycobacterium* JS330 was isolated from soil by selective enrichment with 2NI as a sole carbon, nitrogen, and energy source. The initial step in the degradation pathway was determined to be the hydrolytic denitration of 2NI to produce imidazol-2-one (IM2O) and nitrite. The amino acid sequence of 2NI nitrohydrolase was highly divergent from those of biochemically characterized enzymes, and it confered drug resistance to 2NI when heterologously expressed in *E. coli*. The ability to degrade 2NI in the environment was widespread, indicating a substantial flux of 2NI and its analogs in natural ecosystems. The unusual reaction catalyzed by the novel nitrohydrolase represents the discovery of a previously unreported drug resistance mechanism in soil prior to its identification in clinical situations.

Biodegradation pathways of synthetic nitroaromatic compounds and anilines are well documented, but nothing was known about catabolism of nitroanilines. 5NAA, a natural analog of nitroanilines, is produced by *Streptomyces scabies*. *Bradyrhizobium* JS329 was isolated from

soil by selective enrichment with 5NAA as a sole carbon, nitrogen and energy source. Enzyme assays indicated that the degradation pathway of 5NAA was initiated by 5NAA aminohydrolase (NaaA) to form 5NSA which was then oxidized by 5NSA dioxygenase (NaaB) to open the aromatic ring. The resulting ring-fission product underwent spontaneous formation of lactone intermediates accompanied by the removal of the nitro group as nitrite. The lactone ring was opened by the lactone hydrolase (NaaC) to produce maleylpyruvate. Sequence analysis revealed that *naaA*, *naaB*, and *naaC* were deeply branched. The results provide the first clear evidence for the complete biodegradation pathway of nitroanilines, reveal a novel deamination reaction for aromatic amines, and provide the first example for the denitration mechanism of ring-fission products from nitro aromatic compounds.

Degradation pathways of 5NAA and 2NI serve as precedents for those of nitroaniline and nitroimidazole pollutants. The thesis work supports our hypothesis that the study of the metabolism of natural organic compounds selected on the basis of unusual structural features and ecological roles can reveal new metabolic diversity. The findings also suggest that the discovery of novel metabolic diversity enables functional annotations, and can identify the origin of novel drug resistance mechanisms.

CHAPTER 1

Introduction

1.1 OVERVIEW OF DISSERTATION RESEARCH

Biodegradation mechanisms of naturally occurring nitro compounds, 5-nitroanthranilic acid (5NAA) and 2-nitroimidazole (2NI), were studied on the basis of biochemistry and molecular biology. Chapter 1 is a literature review of biodegradation mechanisms of nitro and amino compounds, mechanisms of action of nitroimidazole drugs, and mechanisms of drug resistance. The work described in Chapter 2 established the initial step in the biodegradation of 2-nitroimidazole by *Mycobacterium* JS330 isolated from garden soil, revealed a deeply-branched gene encoding 2NI nitrohydrolase which catalyzes an unusual reaction, demonstrated that *nnh* confers drug resistance when heterologously expressed in *E. coli*, and determined the environmental distribution of 2NI-degrading bacteria. Chapters 3 and 4 established the biochemistry and genetics of biodegradation of 5-nitroanthranilic acid by *Bradyrhizobium* JS329 isolated from potato farm soil, characterized an unusual aminohydrolase involved in the deamination of 5-nitroanthranilic acid to produce 5-nitrosalicylic acid, and determined the mechanism of denitration from the ring fission product of 5-nitrosalicylate. Chapter 5 summarizes the results and significance of our work, proposes future work and remaining questions.

1.2 RESEARCH OBJECTIVES

The overarching goal of work presented in this thesis seeks to uncover novel metabolic diversity involved in biodegradation of naturally occurring nitro compounds.

The objectives of my thesis work include: 1) discovering new metabolic diversity; 2) elucidating unknown biodegradation mechanisms of nitroanilines and nitroheterocycles; 3) exploring the evolutionary relationship of biodegradation pathways of synthetic compounds and their natural analogs; 4) providing deeper understanding of the environmental reservoir of drug resistance.

1.3 BACKGROUND

Nitro-substituted compounds are widely used as dyes, pesticides, synthetic intermediates, and explosives (e.g. nitroglycerin, TNT, DNT) (98). The research on biodegradation of nitro compounds has focused on synthetic chemicals and a great deal is known about the biochemistry and molecular biology of the pathways. The biodegradation pathways for synthetic compounds recently released into the biosphere appear to have evolved and been assembled by horizontal gene transfer from a variety of catabolic genes already present in nature (47-48, 74). However, our current understanding of natural metabolic diversity is fragmentary and fails to predict the biodegradation pathways for synthetic pollutants. Deeper investigation of metabolic diversity involved in biodegradation of natural nitro compounds has several long-term objectives including: a) the biosynthesis and biodegradation of an array of explosives using green chemistry strategies, b) development of biosensors to determine the distribution of explosive pollutants in the environment, c) rational design of environmentally-friendly synthetic nitro compounds including explosives and antibiotics.

Over 200 natural nitro compounds are produced by a variety of microbes, plants, and animals (Nishino, S. F., J. C. Spain, and R. J. Parry, submitted for publication), but little is known about their biodegradation mechanisms (Fig. 1.1 for examples). Nitro

compounds are formed biologically by direct nitration via attack of reactive species such as peroxynitrite ($ONOO^-$) and nitrogen dioxide (NO_2^+) (5, 11, 39, 86), or from oxidation of an amino group (12, 110). It is clear that such compounds do not accumulate in nature. Therefore, they must be biodegraded and it should be possible to find bacteria with the appropriate catabolic pathways.

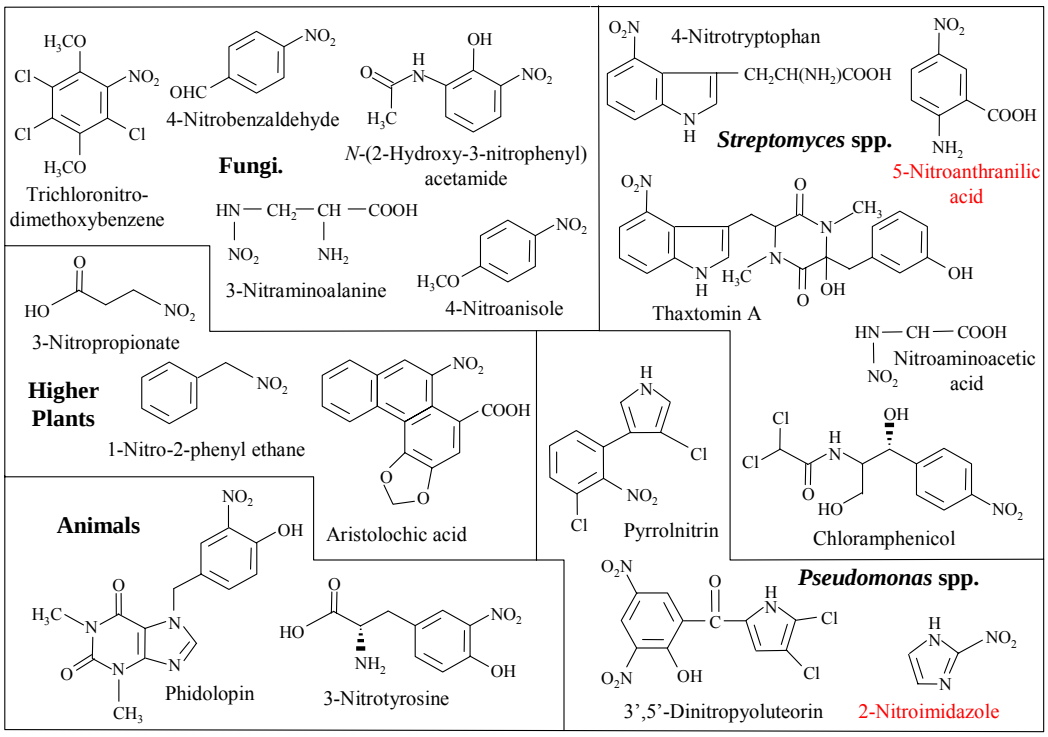

Fig. 1.1 Representative natural nitro compounds identified in animals, plants, and microbes (Courtesy of Dr. Spain).

As part of a larger effort to explore metabolic diversity found in catabolic pathways, 5-nitroanthranilic acid (5NAA), a natural nitroaniline, and 2-nitroimidazole (azomycin, 2NI), a natural nitro heterocyclic antibiotic, were chosen as model compounds for further research (Fig. 1.1). Characterization of the biochemistry and molecular basis of the degradation pathways was conducted in order to shed light on the

biodegradation mechanisms for other nitroanilines and nitroheterocyclic compounds. A second goal was to evaluate whether the genes involved in degradation of natural nitroaromatic compounds can be recruited during evolution of catabolic pathways for synthetic nitroaromatic pollutants.

5NAA is produced by *Streptomyces scabies*, the causative agent for potato scab which causes huge economic losses in agriculture. The bacteria have been isolated from a variety of places including Australia, Germany and Japan, but the physiological role of 5NAA is unclear (54). Synthetic 5NAA is the starting material for synthesis of various nitroaromatic compounds and dyes (7).

Synthetic nitroanilines are toxic (53) and used for the synthesis of pharmaceuticals, dyes and pigments (87) or as explosives (Fig. 1.2) (1). For example, MNA (N-methyl-4-nitroaniline) (Fig. 1.2) is used as a stabilizer for double-base and minimum smoke propellants (1). A few organisms were reported to degrade 3- (87) and 4-nitroaniline (83, 114), but the catabolic pathways of 5NAA and its synthetic nitroaniline analogs were completely unknown prior to the thesis work and such compounds are typically classified as resistant or nondegradable (87). Determination of the degradation pathway of 5NAA will provide insight about the effect of the nitro group on the strategy for degradation and assimilation of nitroaniline pollutants, and will reveal evolutionary origins of the enzymes involved in biodegradation of synthetic nitroanilines that have only been in the biosphere for a short time.

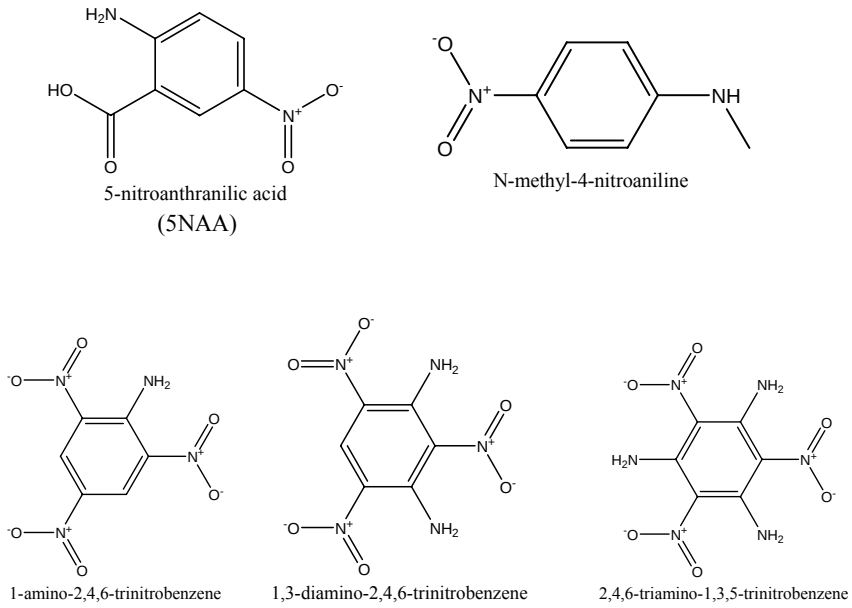

Fig. 1.2 Structures of 5NAA and its explosive analogs.

2NI, produced by bacteria including *Nocardia mesenterica*, *Pseudomonas fluorescens* and *Streptomyces eurocidicus* (73, 93), is an antibiotic that inhibits both bacteria and protozoa (*Trichomonas*) (42). It is representative of a variety of nitroimidazoles with wide application from medical to military field. For example, nitroimidazoles (Fig. 1.3 & Fig. 1.4) are prodrugs, effective against drug resistant *Mycobacterium tuberculosis* (6) and as radiosensitizers for tumor therapy (68). 2NI derivative, benznidazole, is the common drug for the treatment for Chagas' disease (30), and the only one used in Brazil and Argentina (78). Chagas' disease, caused by *Trypanosoma cruzi* (protozoan parasite), is threatening more than 25 million people, mostly in South America, and is infecting approximately 10 million people in the world (http://www.who.int/mediacentre/factsheets/fs340/en/index.html).

In addition, dinitroimidazole (Fig. 1.5), is a powerful, insensitive high explosive, existing as three isomers (2,4-dinitroimidazole, 1,4-dinitroimidazole, and 4,5-

dinitroimidazole), of which 2,4-dinitroimidazole is the best explosive. A great deal is known about the mechanisms of biotransformation of nitroimidazoles (see section 1.7), but nothing was known about their biodegradation mechanism.

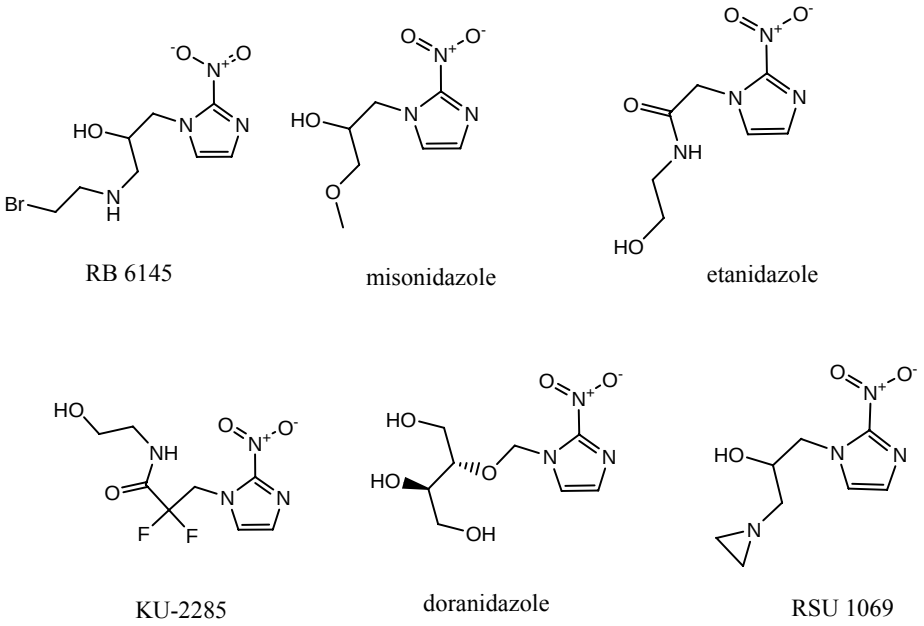

Fig. 1.3 Examples of 2NI analogs developed for cancer treatment (68).

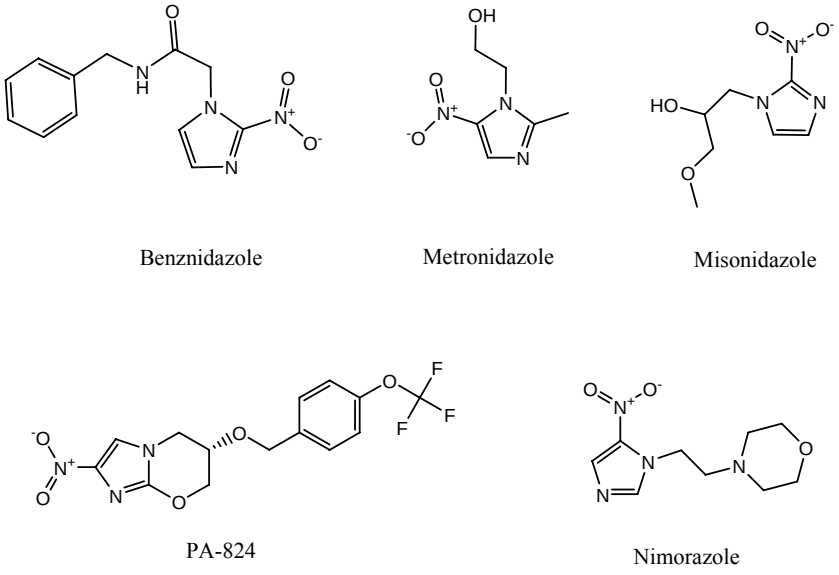

Fig. 1.4 Structures of antibacterial nitroimidazoles (6).

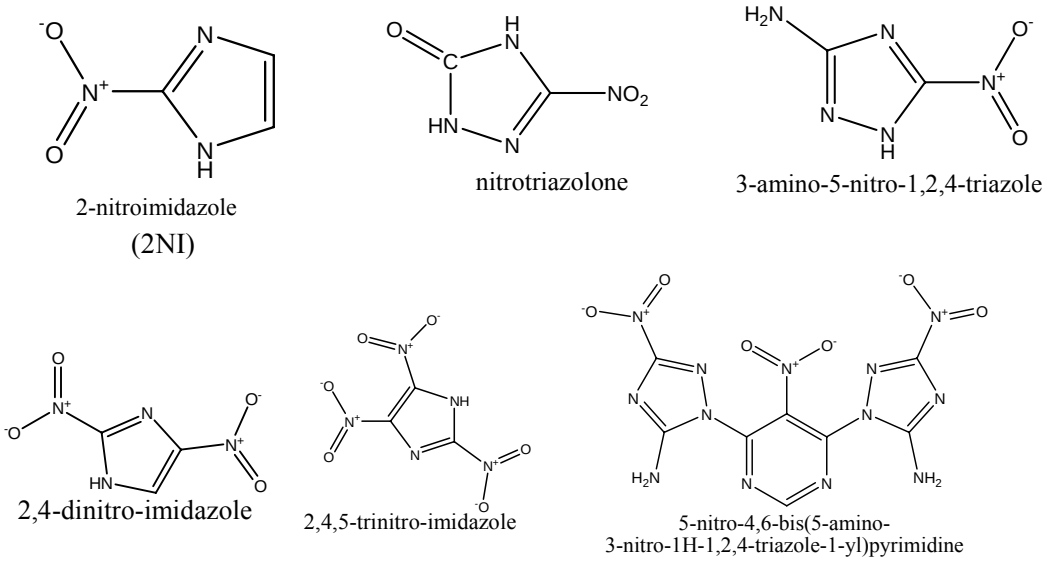

Fig. 1.5 Structures of 2NI and its explosive analogs.

1.4 BIODEGRADATION OF NITRO COMPOUNDS

The compounds chosen for study, 5NAA and 2NI, are nitro compounds with unique structures representative of amino nitroaromatic and nitroheterocyclic compounds. To date, mechanisms of nitro degradation have been extensively studied and the following section provides a discussion of known biodegradation pathways for nitro compounds. The thesis work revealed hydrolytic denitration (See Chapter 2) and nitrite release during lactone formation (See Chapter 4) which were not reported previously.

1.4.1 Biodegradation of nitroaromatic compounds

The initial steps in the biodegradation of nitroaromatic compounds (Fig. 1.6) have been established through a number of studies (49, 71, 97). For non-polar compounds the initial reaction is catalyzed by dioxygenases to eliminate the nitro group as nitrite, whereas for more polar compounds it is catalyzed by flavoprotein monooxygenases to

remove the nitro group as nitrite and replace it with a keto or hydroxyl group. The third possibility is that the nitro group can be reduced by nitroreductases to produce the nitroso and then hydroxylamino intermediates, which are further transformed by mutase, or hydroxylaminolyase enzymes.

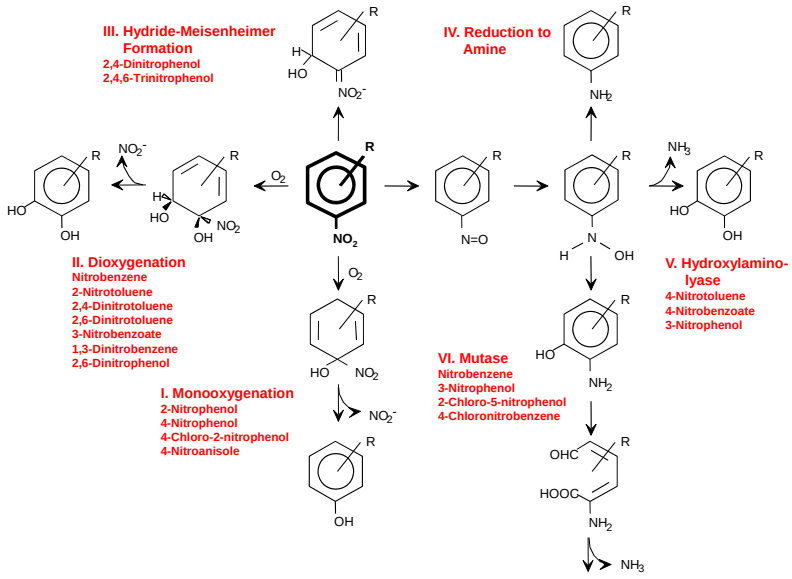

Fig. 1.6 Mechanisms for the transformation of nitro groups (C-NO_2 bond) in synthetic nitro aromatic compounds (98).

The nitrobenzene degradation pathway in *Pseudomonas pseudoalcaligenes* JS45 is initiated by nitrobenzene nitroreductase (NbzA) (8) to form hydroxylaminobenzene (HAB), followed by a structural rearrangement to produce 2-aminophenol catalyzed by a HAB mutase, and subsequently the aromatic ring is opened by a dioxygenase (Fig. 1.7) (72). In addition to the nitrobenzene degraders, *Pseudomonas pseudoalcaligenes* JS45 (8), *Pseudomonas putida* HS12 (76), and *Comamonas* (112), the gene encoding HAB mutase was also found in *Mycobacterium tuberculosis* H37Rv, which does not grow on nitrobenzene. The gene from *Mycobacterium* encoded an enzyme acting on HAB when

expressed in *E. coli* (18). The gene is widespread among mycobacteria, but the physiological role in mycobacteria is still a mystery. Our hypothesis is that HAB mutase in *Mycobacteria* can confer drug resistance by eliminating toxic hydroxylamine intermediates.

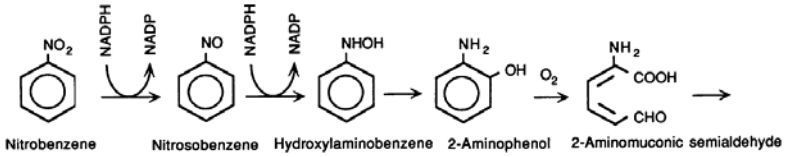

Fig. 1.7 Partial reductive pathways for NB via mutase-catalyzed rearrangements.

Nitroreductase is also responsible for initial nitro group reduction during the biodegradation of 2-nitrobenzoate and 4-nitrobenzoate. But their downstream pathways are divergent: mutase (Fig. 1.8) (66) and hydroxylaminolyase (Fig. 1.9) (31, 35, 43) are involved in lower pathway of 2-nitrobenzoate and 4-nitrobenzoate, respectively.

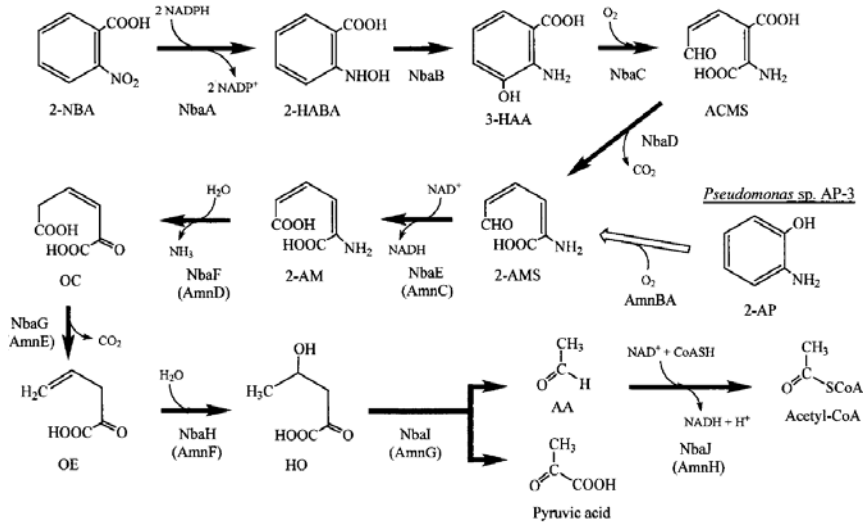

Fig. 1.8 Biodegradation pathway of 2-nitrobenzoate (66).

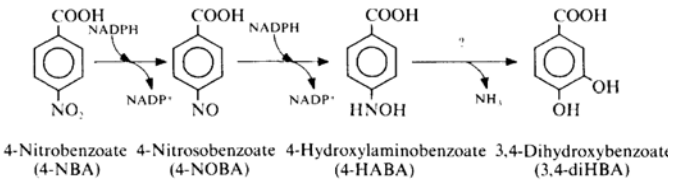

Fig. 1.9 Biodegradation of 4-nitrobenzoate via a hydroxylaminolyase-mediated reaction (31)

In contrast to 2-, 4-nitrobenzoate, biodegradation of 3-nitrobenzoate by *Pseudomonas* sp. strain JS51 is initiated by a dioxygenase to produce protocatechuate and nitrite (67). The enzyme involved is a multi-component dioxygenase, including an oxygenase component and an NAD(P)H-dependent aromatic oxidoreductase component responsible for reducing the oxygenase (81). The interesting question is what causes the different mechanisms of conversion of the isomers: nitroreductase responsible for initial attack of the nitro group from 2-/4-nitrobenzoate but dioxygenase for 3-nitrobenzoate, and mutase responsible for transformation of 2-hydroxylaminobenzoate but hydroxylaminolyase for 4-hydroxylaminobenzoate.

Biodegradation of *p*-nitrophenol has been studied extensively, and the nitro group is eliminated as nitrite prior to ring fission by monooxgenases to produce hydroquinone or hydroxylquinol. In the hydroquinone degradation pathway (Fig. 1.10), a single-component FAD-dependent monooxygenase (PnpA) is responsible for removal of the nitro group accompanied by the formation of *p*-benzoquinone, which is subsequently reduced to hydroquinone by FMN-dependent reductase (PnpB). Both enzymes require NADPH as the electron donor (96, 115). Heterologous overexpression, purification and sequence analysis reveal that PnpA and PnpB from *Pseudomonas* sp. Strain WBC-3 are related to 3-hydroxylphenylacetate hydrolase and NAD(P)H:quinone oxidoreductase (115).

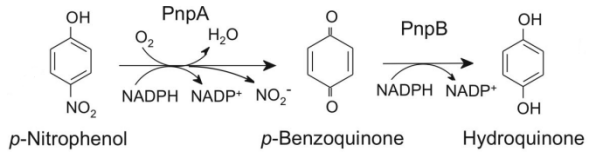

Fig. 1.10 Biodegradation of *p*-nitrophenol in the hydroquinone pathway(115).

In the hydroxylquinol pathway (Fig. 1.11), a two-component flavin-diffusible monooxygenase (NpdA1 and NpdA2) is responsible for the elimination of the nitro group as nitrite (46, 50, 77). NpdA1 is the reductase component to produce $FADH_2$ with NADH as the electron donor; and NpdA2 is the oxygenase component involving in removal of the nitro group as nitrite with $FADH_2$ as the electron donor. The immediate product, *p*-benzoquinone, was identified only recently (61). Sequence analysis revealed that NpdA2 from most of *p*-nitrophenol degraders are phylogenetically related to each other and to chlorophenol monooxygenases (61). Alternatively, PNP can be transformed to 4-nitrocatechol prior to the attack by NpdA1A2 (46, 50, 77), but the genetic basis is still unknown.

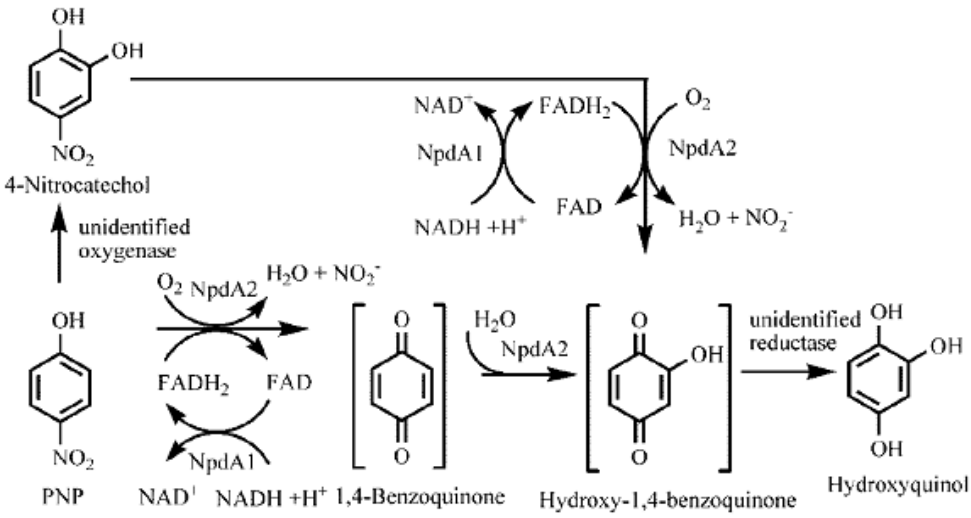

Fig. 1.11 Biodegradation of *p*-nitrophenol in the hydroxylquinol pathway (77).

The hydroquinone degradation pathway exists in *Moraxella* sp. (99), and *Pseudomonas* sp. strain WBC-3 (115); whereas the hydroxylquinol pathway exists in *Bacillus* (50), *Rhodococcus* (55, 100), *Arthrobacter* (61, 77). Interestingly, the hydroquinone degradation pathway and hydroxylquinol pathway are mainly present in gram-negative and gram-positive bacteria, respectively. It remains to be determined what causes the difference: an accident of evolution or some detail of bacterial physiology that constrains the options.

The nitro group of nitrophenols is commonly believed to be eliminated as nitrite by a monooxygenase prior to ring fission as discuss above (71). However, nitrophenols that are intermediates in the 2,6-DNT, picric acid (2,4,6-trinitrophenol) and 5NAA (See Chapter 3) pathways are exceptions. Biodegradation of 2,6-DNT is initiated by a dioxygenase to form 3-methyl-4-nitrocatechol (3M4NC) with the release of nitrite, followed by ring cleavage without prior nitro removal. But the nitro group of the resulting nitro aliphatic compound HNPA (2-hydroxy-5-nitropenta-2,4-dienoic acid) (Fig. 1.12) is removed by an unknown mechanism (69). The thesis work presented herein reveals two previously unreported denitration mechanisms: hydrolytic denitration (See Chapter 2) and spontaneous nitro removal during lactone formation (See Chapter 4). Therefore, it remains to be seen whether either mechanism can apply to the denitration of HNPA (Fig. 1.12).

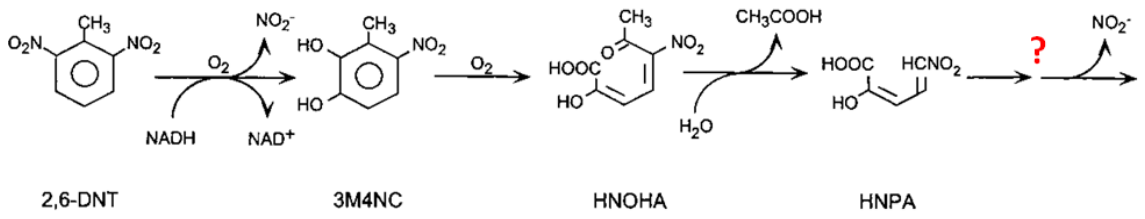

Fig. 1.12 Biodegradation pathway of 2,6-DNT (69).

The degradation of 2,4,6-trinitrophenol (picric acid) by *Nocardioides simplex* FJ2-1A, is initiated by the hydride ion-mediated reduction of the aromatic ring to form a Meisenheimer intermediate and subsequent release of nitrite, catalyzed by F420 dependent enzyme system, followed by hydrolytic ring fission to produce 4,6-dinitrohexanoate (4,6-DNH) (Fig. 1.13). The reduction is catalyzed by a two-component enzyme system: an NADPH-dependent F420 reductase; and a transferase that catalyzes transfer of a hydride from reduced F420 to the aromatic ring of picric acid (19). The reactions involved in the removal of the nitro groups from the aliphatic ring fission product (4,6-DNH) are unknown. 4,6-DNH is the structural analog of 3-nitropropionic acid (See Fig. 1.17 below), so it remains to be seen whether the biodegradation mechanism of 3-nitropropionic acid reported recently (70) can serve as a precedent for that of 4,6-DNH.

Knackmuss and colleagues examined the degradation of picric acid (37) and identified the relevant genes in *Rhodococcus (opacus) erythropolis* (Fig. 1.14 & Fig. 1.15). Identification of key genes *npdI* (encoding hydride transferase II), *npdG* (encoding NADPH-dependent F420 oxidoreductase), and *npdC* (encoding hydride transferase I) provided the genetic basis and further confirmed the mechanism of initial reactions proposed in previous studies (Fig. 1.13) (19).

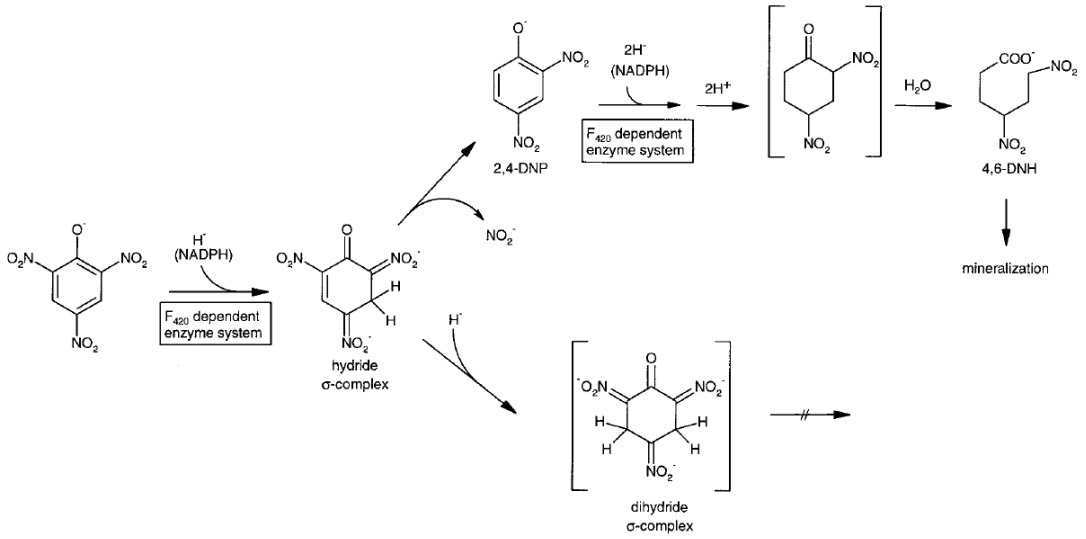

Fig. 1.13 Proposed initial degradation pathway of picric acid by *Nocardioides simplex* FJ2-1A (19).

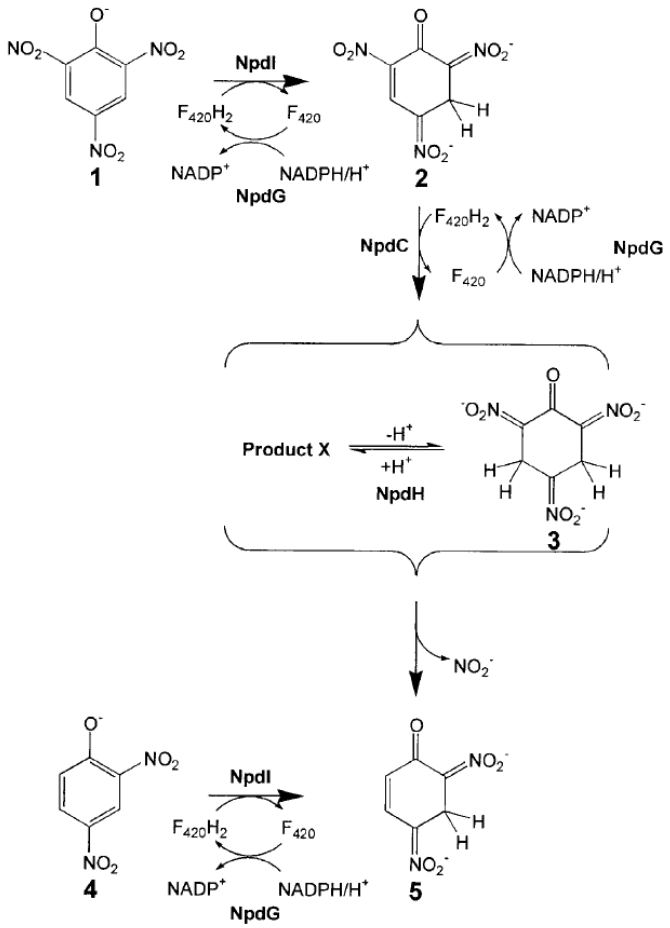

Fig. 1.14 Biodegradation pathway of picric acid by *R. (opacus) erythropolis* HL PM-1 (37).

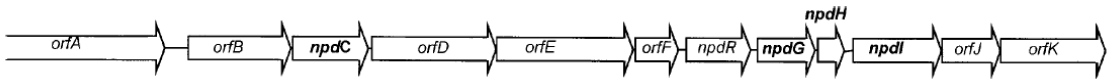

Fig. 1.15 The gene cluster involved in the biodegradation of picric acid by *R. (opacus) erythropolis* HL PM-1 (37). *npdC* encodes hydride transferase I ; *npdG* encodes NADPH-dependent F420 oxidoreductase; *npdI* encodes hydride transferase II.

In summary, the biodegradation pathways for nitroaromatic compounds have been studied extensively, and the general rule is: the aromatic ring is activated by hydroxylation catalyzed by oxygenases to produce the key intermediates (catechols, homoprotocatechuate, gentisate), followed by ring cleavage catalyzed by dioxygenases,

and the resulting aliphatic compounds feed into the TCA cycle (58). Alternatively, the nitro group is reduced to the corresponding hydroxylamine which is channeled into productive pathways. Both mechanisms require a significant input of electrons from NAD(P)H.

1.4.2 Biodegradation of nitroaliphatic compounds

Much less is known about degradation of nitroaliphatic compounds because bacteria able to use them as growth substrates have not been isolated until very recently (70). Nitroalkanes exist as either neutral nitroalkanes or anionic alkyl nitronates under normal conditions (Fig. 1.16) (64).

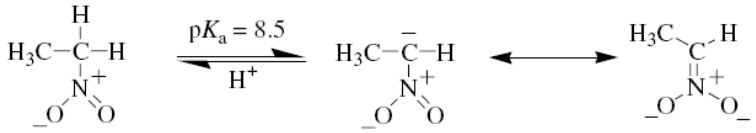

Fig. 1.16 The interconversion of neutral nitroethane (left) and anionic ethylnitronate (right) in solution (64).

Nitropropane is oxidized by nitronate mono-oxygenases (NMO, E.C. 1.13.12.16, formerly 2-nitropropane dioxygenase), to acetone with the formation of nitrite. NMOs contain a flavin cofactor and aspartate to remove the nitro group and abstract the proton from the C-H bond adjacent to the nitro group. The nitroalkane monooxygenation activity has been found in bacteria, fungi and plants (23), but its physiological role is unknown.

Recently Nishino and colleagues reported the biodegradation pathway for 3-nitropropionic acid (Fig. 1.17), and the initial reaction was proposed to be catalyzed by a monooxygenase that converted 3-nitropropionic acid into malonate semialdehyde with simultaneous release of nitrite (70). The gene is part of a clade within COG2070, many of

the members of which were misannotated as encoding 2-nitropropane dioxygenases based on weak sequence similarity. The results provide the first insight about the physiological role of the enzymes that attack nitroaliphatic compounds.

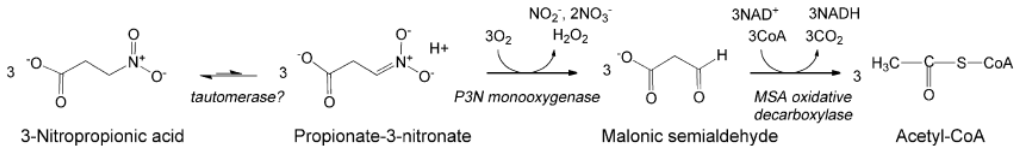

Fig. 1.17 Biodegradation pathway of 3-nitropropionic acid (70).

Nitroalkanes can also be oxidized by nitroalkane oxidase (NAO, EC 1.7.3.1) via the reaction: nitroalkane + O_2 + H_2O → aldehyde or ketone + nitrite + H_2O_2. Neutral nitroalkanes are the preferred substrates for NAO, a flavin-N(5)-substrate adduct is formed transiently, and H_2O_2 is produced during the reaction. In contrast, anionic nitronates are the preferred substrates for NMO, an anionic flavin is formed, and H_2O_2 production is negligible (29, 62).

1.4.3 Biodegradation of synthetic N-NO_2 compounds

RDX (hexahydro-1,3,5-trinitro-1,3,5-triazine) is a heterocyclic nitramine compound which is used as an explosive. Most of RDX-degrading bacteria utilize RDX as a sole nitrogen source (10, 15), but Thompson et al. isolated *Williamsia* sp. KTR4 and *Gordonia* sp.KTR9 able to grow on RDX from a military site, the first report that RDX can serve as a carbon, nitrogen and energy source (103).

The biodegradation pathway of RDX was not established until Fournier's work (24). The enzyme involved in the initial step of biodegradation of RDX by *Rhodococcus* DN22 is related to the rabbit liver cytochrome P450 2B4 (9). Cytochrome P450 2B4 catalyzes sequential transfer of two single electrons to RDX to produce compound I and

subsequently compound II accompanied by formation of nitrite. Compound II then undergoes spontaneously hydrolytic ring-fission to yield nitroaliphatic compound NDAB (4-nitro-2,4-diazabutanal) (Fig. 1.18) (9). Recent work suggests that biodegradation of RDX by *Gordonia* KTR9 doesn't require a specific transporter for the uptake of RDX as the nitrogen source, and exogenous nitrogen sources, ammonia, nitrite and nitrate, can significantly inhibit its biodegradation activity of RDX (44).

xplA (homologous to cytochrome P450) and *xplB* (homologous to adrenodoxin reductase) were determined to be responsible for the denitration of RDX in *Rhodococcus rhodochrous* (90). The identification of the key RDX-degrading genes provided the basis for Bruce group's later work on biochemical characterization of the enzymes and bioremediation of RDX (45, 85). *xplAB* from *Rhodococcus rhodochrous* 11Y and *Microbacterium,* two phylogenetically and geographically distinct isolates, are almost identical and carried by plasmids, which indicates the important role of horizontal gene transfer for the biodegradation of RDX (4). The evolutionary origins of *xplAB* are unknown.

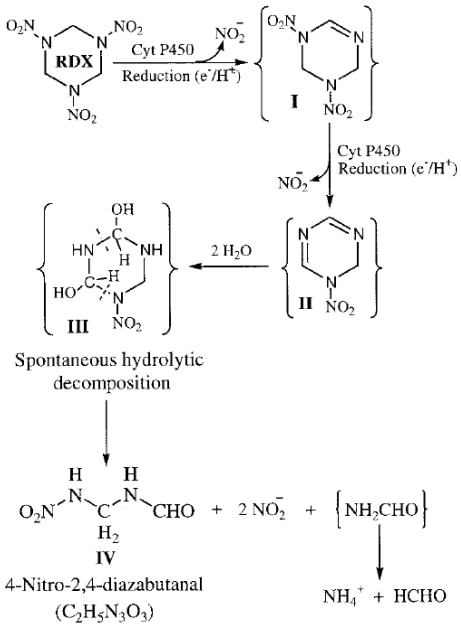

Fig. 1.18 RDX biotransformation pathway (9).

In addition to RDX, the nitro group of other N-NO_2 compounds such as N-nitrosodimethylamine (NDMA) (Fig. 1.19), is also released by cytochrome P450 with the formation of a double bond and nitrite (105). Cytochrome P450, absorption wavelength near 450 nm, is a diverse superfamily of hemoproteins found in a variety of microorganisms, and catalyses various chemical reactions including oxidation, reduction, ester cleavage, ring formation, dehydration, desaturation and isomerization (33).

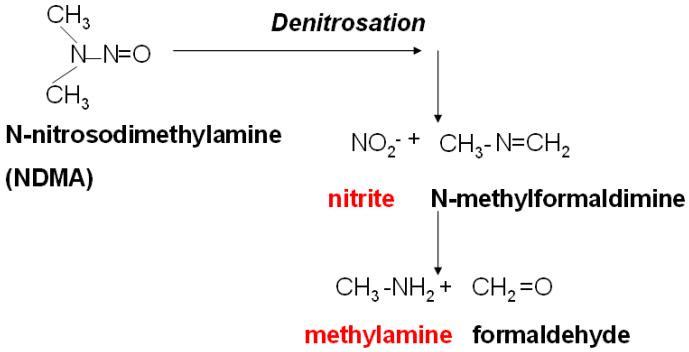

Fig. 1.19 Denitrosation of NDMA.

1.5 BIODEGRADATION OF AMINO COMPOUNDS

In addition to nitro group removal, complete mineralization of the model compound 5NAA requires removal of the amino group. Deamination reactions for a number of natural and synthetic compounds (anilines) (described below) have been extensively characterized. But hydrolytic deamination from amino phenyl compounds (See Chapter 3 and 4) was not previously reported.

1.5.1 Deamination by dioxygenases (typical for anilines)

Synthetic anilines, widespread pollutants in a variety of systems, are toxic to mammals (14) and fish (38). Under anaerobic conditions, aniline can be reductively deaminated with release of ammonia (88, 104). In bacteria that grow on aniline under aerobic conditions, a dioxygenase oxidizes aniline to catechol with formation of ammonia (Fig. 1.20) (27, 59, 106). Aniline dioxygenase is a multi-component enzyme (TdnA1A2B) (26, 28), and distantly related to other Reiske non heme iron oxygenases (108). TdnQ, similar to glutamine synthetase, is also required for the oxidative deamination of aniline to catechol (27), but its physiological role is still unknown, so determination of the function of TdnQ can deepen our understanding of the mechanism of deamination.

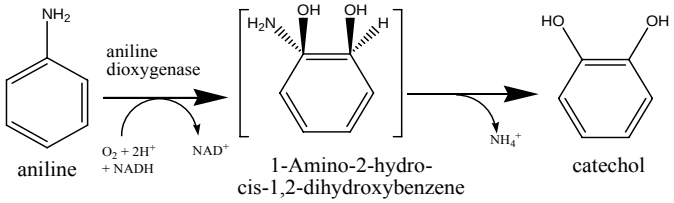

Fig. 1.20 Deamination of aniline catalyzed by a dioxygenase (Modified from University of Minnesota Biocatalysis/Biodegradation Database, http://umbbd.msi.umn.edu/core/graphics/r1047.gif).

5NAA is a nitro substituted analog of anthranilate (2-aminobenzoate) which is an important intermediate in the biodegradation and biosynthesis of tryptophan (36, 57, 101). Anthranilate is transformed to catechol by a dioxygenase in *Pseudomonas* (56) or *Burkholderia cepacia* DBO1 (13), or into 2,3-dihydroxybenzoate by a flavoprotein hydroxylase in the yeast *Trichosporon cutaneum* (80). But the exception is 3-hydroxyanthranilate, an intermediate of 2-nitrobenzoate biodegradation, whose amino group is removed by a hydrolytic deaminase (EC 3.5.99.5) after the aromatic ring is opened by 3-hydroxylanthranilate 3,4-dioxygenase (Fig. 1.8) (66).

1.5.2 Deamination by transaminases (typical for amino acids)

The amino group from amino acids can be eliminated as ammonia by transaminases (aminotransferases). The enzyme can catalyze transfer of the amino group from amino acids to α-keto acids such as α-ketoglutarate, oxaloacetate, or pyruvate, resulting in the formation of another α-keto acid and amino acid (Fig. 1.21 for example). Pyridoxal phosphate is the required cofactor of such enzymes.

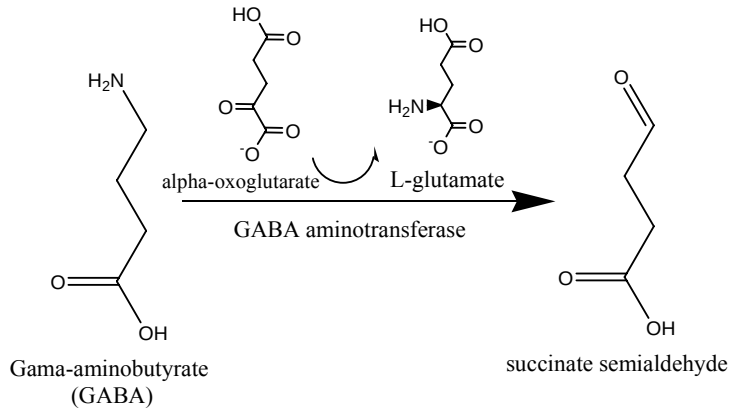

Fig. 1.21 Mechanism of deamination of Gama-aminobutyrate (3).

1.5.3 Amine oxidases

Amine oxidases catalyze the oxidative deamination reaction with monoamines or diamines as substrates shown below:

$R\text{-}CH_2NH_2 + O_2 + H_2O \rightarrow R\text{-}CHO + NH_3 + H_2O_2$

Amine oxidases (monoamine oxidases, EC. 1.4.3.4; diamine oxidases, EC. 1.4.3.6) contain flavin or copper as a cofactor. In this type of reaction, the alpha C-H bond is cleaved and the amino group is replaced by oxygen, resulting in the formation of aldehyde, ammonia and hydrogen peroxide (113). Putrescine (diamine with 4 C-atoms apart) oxidase from *Rhodococcus erythropolis* contains a Glu within the substrate binding site and FAD for catalysis. Substrates containing two amino groups are crucial for the enzymatic activity (107). Compared to flavin-containing amine oxidases, copper amine oxidases have a different catalytic mechanism in spite of the same reaction type. This type of enzymes contains copper, TPQ (2,4,5-trihydroxyphenylalanine quinine, derived from the posttranslational modification of tyrosine), and Ca^{2+}. Cu^{2+}, coordinated by conserved histidines, plays the role of activating oxygen; whereas TPQ is essential for the release of amino group from aliphatic amines (95). The interesting question is why the amino group from amino acids and aliphatic mono-/di-amines are removed by transaminases and amine oxidases, respectively. Our hypothesis is that the carboxyl group of amino acids inhibits the activity of amine oxidases, as is seen with putrescine oxidase that is highly active against putrescine (1,4-butanediamine), but slightly active against L-ornithine (107). The mechanism responsible for the dramatic difference of activity of putrescine oxidase against the two structural analogs remains to be determined.

1.5.4 Deamination by hydrolases (typically for aliphatic/heterocyclic compounds)

It is well established that hydrolases are involved in the deamination of heterocyclic amino compounds such as adenosine (52), cytosine (79) and 4-aminoimidazole. 4-Aminoimidazole (Fig. 1.22) is involved in the purine degradation pathway, where it is converted into 4-imidazolone by 4-aminoimidazole deaminase, followed by ring cleavage to produce formiminoglycine. The deaminase was purified from *Clostridium cylindrosporum*, and required Fe^{2+} as the cofactor (25, 84).

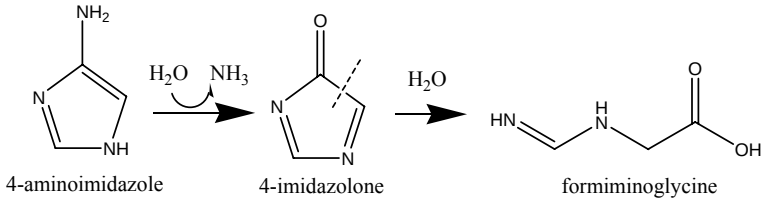

Fig. 1.22 Biodegradation of 4-aminoimidazole.

In the last two decades, the biodegradation of a second heterocyclic amine, melamine (2,4,6-triamino-*s*-triazine), was established. Melamine contains a heterocyclic aromatic ring with three interspaced carbon and nitrogen atoms (Fig. 1.23). It is transformed by bacteria into cyanuric acid via the intermediates, ammeline and ammelide, by three hydrolytic deamination steps (Fig. 1.23) (89, 91-92). Melamine deaminase (TriA) expressed in *E. coli*, catalyzes the hydrolytic deamination of melamine and ammeline (89). TriA is 98% identical to atrazine chlorohydrolase (AtzA), but TriA and AtzA are specific for deamination of melamine and dehalogenation of halo-substituted triazines (89). The hydrolytic deaminases typically contain a zinc-binding domain (22). There are no previous reports of hydrolytic deamination for amino phenyl compounds.

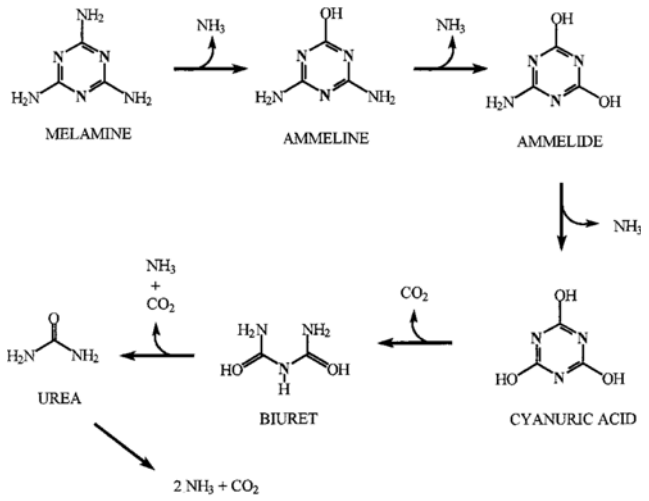

Fig. 1.23 Hydrolytic deamination of melamine (92).

1.6 RING-CLEAVAGE OF 5-SUBSTITUTED SALICYLATE

5-Nitrosalicylate (5NSA), the deamination product of 5NAA, is oxidized by a ring-cleavage dioxygenase prior to removal of the nitro group as nitrite (See Chapter 3). The mechanism of nitro group removal from the ring-fission product was a mystery, but the analogous reaction for 5-halosalicylate was well established.

Salicylate (2-hydroxybenzoate) and gentisate (2,5-dihydroxybenzoate) are the key intermediates in the biodegradation of naphthalene (32). Biodegradation of gentisate is initiated by a ring-cleavage gentisate 1,2-dioxygenase (32). An enzyme from *Pseudaminobacter salicylatoxidans* with 28% identity to gentisate 1,2-dioxygenase from *Pseudomonas alcaligenes* NCIMB 9867 oxidizes salicylate and a variety of substituted salicylates, but not 5-nitrosalicylate (5NSA) (41). The gentisate/salicylate 1,2-dioxygenase requires Fe^{2+} and contains a conserved histidine pair essential for anchoring ferrous iron in the catalytic center (40).

Oxidative ring-cleavage of 5-halosalicylates, produces lactones with the simultaneous release of HX (X: halide). The lactones are further hydrolyzed to maleylpyruvate (Fig. 1.24). Lactones are the first products that can be detected after the dioxygenation of 5-halosalicylates by *Pseudaminobacter salicylatoxidans* (41).

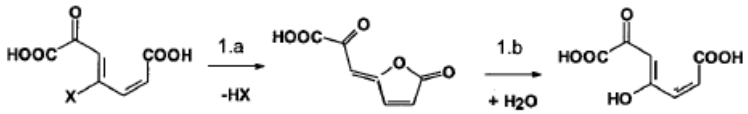

Fig. 1.24 Proposed consecutive abiotic reactions for the dioxygenation products of halogenated salicylates (41).

From the above discussion it is clear that a variety of 5-substituted salicylates can be oxidized by enzymes analogous to gentisate or salicylate 1,2-dioxygenases. The halogen is released during lactone formation. The reaction serves as a good precedent for that of the nitrite release during the biodegradation of 5NSA (See Chapter 4).

1.7 METABOLISM OF NITROIMIDAZOLES

Nitroimidazoles including 2NI, metronidazole, benznidazole, and PA-824, are clinically important antibiotics (6, 30, 68). Nothing was known about the biodegradation mechanism of nitroheterocycles including nitroimidazoles, but the biotransformation mechanisms of nitroimidazoles have been well studied. Biotransformation is a biological modification of one chemical to another (transformed), whereas biodegradation is the microbial destruction of a chemical compound to serve as a source of carbon and energy for the growth of microbes.

The antibiotic mechanism of nitroimidazole prodrugs involves enzymatic reduction (63, 82) of the nitro group to the corresponding toxic hydroxylamine or nitroso

derivatives, which in turn react with intracellular nucleophiles including DNA (Fig. 1.25) (6, 68). F420 (deazaflavin cofactor) is a two-electron donor (109), so F420-dependent nitroreductase (Ddn) is insensitive to oxygen (98). In contrast, when a single-electron transfer system is involved in the reduction of nitroimidazoles, the intermediate nitro radical anion is sensitive to oxygen. In the presence of oxygen, the electron carried by the nitro radical anion can be abstracted by O_2, resulting in formation of superoxide which is converted to hydrogen peroxide by superoxide dismutase. The hydrogen peroxide can either be removed by catalase or react with Fe^{2+} via the Fenton reaction to eliminate its toxicity (6). Therefore, hypoxic conditions are typically required for activation of nitroimidazole prodrugs in mammals.

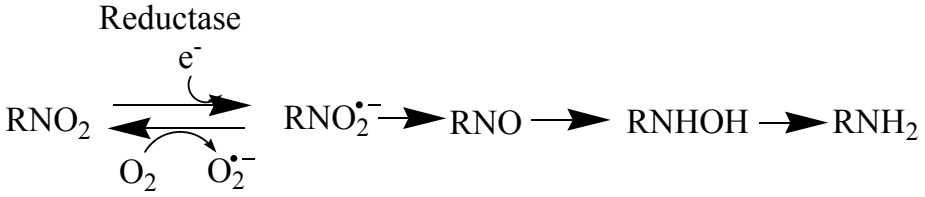

Fig. 1.25 The antibiotic mechanism of 2-nitroimidazoles (68).

1.8 MECHANISMS OF DRUG RESISTANCE

Synthetic antibiotics including nitroimidazoles were deployed within the past century, but resistant strains were observed very rapidly. For example, sulfonamide was put into use in 1930s, and resistance was observed in 1940s (75). The observation indicated that the environment provides the reservoir for drug resistance genes, which was confirmed by recent studies (16-17).

The major antibiotic-resistance mechanisms in bacteria include creation of an impermeable barrier (20), efflux pumps (111), point mutations resulting in failure to activate prodrugs (6) or changes in drug targets (102), and enzymatic inactivation of antibiotics (biotransformation (21, 34, 65, 94) or biodegradation (34, 60)).

Antibiotic-resistance mechanisms other than biodegradation of antibiotics involve fitness trade-offs (51). Bacteria have been isolated for the ability to degrade antibiotics including chloramphenicol, streptomycin and penicillin (2, 60), but little is known about physiological roles of their catabolic enzymes and the evolutionary origin of drug resistance. In Chapter 2, we studied the biodegradation mechanism of 2NI in depth, and discovered a previously unreported drug resistance mechanism.

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CHAPTER 2

Catabolic Pathway for 2-Nitroimidazole Involves a Novel Nitrohydrolase that also Confers Drug Resistance

2.1 ABSTRACT

Antibiotic resistance in pathogens can be mediated by catabolic enzymes thought to originate from soil bacteria, but the physiological functions and evolutionary origins of the enzymes are poorly understood. 2-Nitroimidazole (2NI) is a natural antibiotic and an analog of the synthetic nitroimidazoles for treatment of tuberculosis and cancer. *Mycobacterium* sp. JS330 was isolated from soil based on its ability to use 2NI as a sole growth substrate. The initial step in the degradation pathway is the hydrolytic denitration of 2NI to produce imidazol-2-one (IM2O) and nitrite. The amino acid sequence of 2NI nitrohydrolase is highly divergent from those of biochemically characterized enzymes, and it confers resistance to 2NI when it is heterologously expressed in *E. coli*. The unusual enzymatic reaction seems likely to determine the flux of nitroimidazole in natural ecosystems and also represents the discovery of a previously unreported drug resistance mechanism in soil prior to its identification in clinical situations.

2.2 INTRODUCTION

Many of the small bioactive compounds produced in the biosphere function as attractants, inhibitors, repellants or signals in the complex interactions among biological systems. The biodegradation of such molecules significantly alters the range, intensity, and duration of a chemical signal or renders a chemical defense less effective. Therefore, if an organic compound plays an important ecological role, the bacteria that degrade it play an equally important ecological role by controlling the flux and effects of the compound (23).

2-Nitroimidazole (2NI, azomycin) is produced by soil bacteria including *Nocardia mesenterica*, *Pseudomonas fluorescens* and *Streptomyces eurocidicus* (32, 39). 2NI inhibits both bacteria and protozoa (*Trichomonas*) (21) and is representative of a large group of synthetic nitroimidazole antibiotics used for the treatment of cancer (27) and tuberculosis (4). Estimated annual worldwide use of nitroimidazoles for medical purposes has historically been in the range of 25 to 100 kg per 100,000 global inhabitants (44). Nitroimidazoles are prodrugs that are activated by a variety of reductases (26, 34, 40) to the corresponding hydroxylamino and/or nitroso derivatives, which in turn react with intracellular nucleophiles including DNA (4, 27). They are particularly useful for drug resistant *Mycobacterium tuberculosis* (4) and as radiosensitizers for tumor therapy (27).

As part of our effort to expand the understanding of metabolic diversity, we have isolated a strain of *Mycobacterium* that grows on 2NI as the sole carbon, nitrogen and energy source. The initial step is a previously unreported hydrolytic denitration catalyzed by an enzyme with an amino acid sequence highly divergent from previously characterized enzymes. The enzyme confers resistance to 2NI when expressed heterologously in *E. coli*.

2.3 MATERIALS AND METHODS

2.3.1 Isolation of 2NI degrading bacteria

Enrichments were done in nitrogen-free minimal medium (BLK) (6) at pH 7.2 with 2NI (50 μM) as the sole carbon and nitrogen source. A variety of soil/water samples from several locations in the US were used as the inocula. 2NI concentrations in the liquid enrichment were measured by HPLC (high-performance liquid chromatography) at appropriate intervals. When biodegradation was detected, several subsequent additions of 2NI and transfers to fresh media with increasing concentrations of 2NI led to a highly enriched mixed culture. Isolates were obtained by spreading samples of the enrichment on BLK agar (1.5%) plates containing 2NI (500 μM) as the carbon and nitrogen source.

2.3.2 Identification of 2NI degrading bacteria

Cultures grown in 1/4 strength tryptic soy broth at 30°C were collected for the extraction of genomic DNA with an UltraClean Microbial DNA Isolation Kit (MO BIO, Carlsbad, CA). The 16S rRNA gene was PCR amplified with primers I-341f and I-926r (Table 2.1) (46). Purified PCR products were sequenced by Genewiz (New Jersey) or Nevada Genomics Center (Reno, NV). The 16S rRNA gene sequences (500 bp) were searched against the NCBI database (http://www.ncbi.nlm.nih.gov/) using the BLASTN program (3).

2.3.3 Growth of 2NI degrading bacteria

Isolates were routinely grown on BLK (7) supplemented with 2NI (500 μM) at 30˚C and pH 7.2. Glucose (5 mM) or pyruvate (10 mM) was used as the primary growth substrate with 2NI (500 μM) as the nitrogen source when larger biomass was required.

2.3.4 Analytical methods

The concentrations of 2NI, 2-aminoimidazole, and imidazol-2-one (IM2O) were determined with an Agilent Eclipse XDB-C_{18} column (4.6 mm by 150 mm; 5 μm). The mobile phase for 2NI analysis consisted of a mixture of 95% part A (13.5 mM trifluoroacetic acid in water) and 5% part B (6.75 mM trifluoroacetic acid in acetonitrile) at a flow rate of 0.5 ml/min over 10 min. 2NI was monitored at 325 nm and a retention time of 5.8 min. The mobile phase for 2-aminoimidazole was 98% part A and 2% part B at a flow rate of 0.5 ml/min over a 7-min period. 2-Aminoimidazole was monitored at 220 nm and a retention time of 4.5 min. The mobile phase for imidazole-2-one (IM2O) was 100% water at a flow rate of 0.5 ml/min over 10 min. IM2O was monitored at 205 nm and a retention time of 4.7 min. Analyses of ammonia and nitrite concentrations were based on the methods reported previously (33). Protein was measured with a Pierce (Rockford, IL) BCA (bicinchoninic acid) protein assay reagent kit.

2.3.5 Chemicals

2-Nitroimidazole and 4-nitroimidazole were obtained from Sigma-Aldrich (Milwaukee, WI). Imidazole was from USB corporation (Cleveland, OH). Imidazol-2-one was from Focus Synthesis LLC (San Diego, CA).

2.3.6 Gene library construction and screening

The genomic library of *Mycobacterium* JS330 was constructed as described previously (33). Clones from the genomic library were grown in 96-well plates containing LB medium supplemented with chloramphenicol (12.5 μg/ml) and 1x Fosmid CopyControl Induction Solution (Epicentre Biotechnologies, Madison, WI). After growth, the library was replica plated onto BLK liquid media supplemented with 2NI (100 μM) and 1x Fosmid CopyControl Induction Solution, then incubated overnight at 30˚C. Clones were screened colorimetrically for nitrite release from 2NI.

2.3.7 Identification of *nnh*

The DNA of fosmid pJS900 (Table 2.1) was digested with EcoRI/BamHI, EcoRI/HindIII, PstI/SalI, XbaI/SalI or PstI/BamHI and ligated into a pUC19 vector. The ligation products were transformed into chemically competent *E. coli* NEB 5-alpha according to the manufacturer's protocol (New England Biolabs, Ipswich, MA). The resulting subclones were screened for the ability to release nitrite from 2NI. pJS901 contained gene(s) that encoded enzymes that catalyzed release of nitrite and was sequenced by primer walking starting with M13F and M13R primers (Table 2.1) synthesized by Integrated DNA Technologies (Coralville, IA). The open reading frame (ORF) of 2NI nitrohydrolase was identified with an online ORF finder tool (http://www.ncbi.nlm.nih.gov/gorf/gorf.html).

2.3.8 Fosmid DNA sequencing

Fosmid DNA of pJS900 was purified from the *E. coli* host with the FosmidMAX DNA Purification Kit (Epicentre Biotechnologies, WI) and sent to the Emory GRA Genomic Center for sequencing (http://www.corelabs.emory.edu/labs/gra_genome_center/index.html). The open reading frames (ORFs) of the assembled contigs were identified by GeneMark (5) (http://exon.biology.gatech.edu/) and the amino acid sequence of each ORF was compared to the GenBank database by the BLASTP program (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

2.3.9 Sequence analysis

Multiple sequence alignment was performed by ClustalW in BioEdit. A phylogenetic neighbor joining tree (37) was constructed with the MEGA4 program (43) with bootstrap analysis. The conserved domains of 2NI nitrohydrolase were searched against the Conserved Domains Database (CDD) (25) via the NCBI website (http://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml).

2.3.10 Cloning of 2NI nitrohydrolase

nnh from *Mycobacterium* JS330 was amplified by PCR with the primer pair 2NIDenF and 2NIDenR (Table 2.1) from Integrated DNA Technologies (Coralville, IA). The PCR-amplified 2NI nitrohydrolase DNA fragment was inserted into pET-21a vector at the sites of NdeI and HindIII. The resulting ligation product was transformed into *E. coli* NEB 5-alpha (Table 2.1) according to the protocol of the supplier (New England Biolabs, Ipswich, MA). The

transformants were screened by PCR. pJS902 was subsequently transformed into *E. coli* Rosetta 2(DE3)pLysS Competent Cells (Table 2.1) according to the manufacturer's protocol (Novagen).

2.3.11 Enzyme assays

Extracts prepared from cells of *E. coli* pJS901 or pJS902 were used for the enzyme assays. *E. coli* pJS901 was grown in LB medium supplemented with ampicillin (100 μg/ml) and isopropyl β-D-1-thiogalactopyranoside (IPTG, 200 μg/ml) at 30°C with shaking for 17 hours. *E. coli* pJS902 was grown in LB medium supplemented with D-glucose (1%) and ampicillin (100 μg/ml) at 37°C with shaking until mid-log phase (OD_{600}=0.5-0.8). IPTG (200 μg/ml) was added and the culture was incubated at 24°C for 12 hours.

Cells were harvested by centrifugation, washed twice with ice-cold potassium phosphate buffer (pH 7.0, 20 mM), suspended in the same buffer, then passed twice through a French pressure cell (20,000 psi). The cell lysate was clarified by centrifugation (20,000 x g, 4°C, 20 min). For some experiments, the cell extract was ultrafiltered by centrifugation (Microcon Centrifugal Filter Devices, molecular weight cut-off: 10 kDa) at 4°C, followed by washing twice with phosphate buffer (20 mM, pH 7.0) and the resulting retentate was suspended in the same phosphate buffer. The enzyme assays were carried out at 24°C or 30°C in potassium phosphate buffer (pH 7.0, 20 mM) containing 0.1-1.5 mg of protein/ml and 2NI (100-200 μM). Concentrations of substrates and products were determined by HPLC or colorimetric assays at appropriate intervals. Trifluoroacetic acid (TFA) was added to the reaction mixture (1:100, vol/vol) to stop the reaction, and the acidified reaction mixture was clarified by centrifugation before HPLC analysis or nitrite assay.

2.3.12 Respirometry

Oxygen uptake was measured polarographically at 25°C with a Clark-type oxygen electrode connected to a YSI model 5300 biological oxygen monitor. The cell extract of pJS901 was used for the oxygen uptake analysis polarographically for 2NI denitration.

2.3.13 Drug resistance in *E. coli*

E. coli harboring pUC19 or pJS901 (Table 2.1) was cultured in LB medium supplemented with ampicillin (100 μg/ml) and IPTG (200 μg/ml) at 30°C for 17 hours with shaking. A small portion (50 μl) was transferred to 2.9 ml of liquid medium as above containing various concentrations of 2NI and incubated at 30°C with shaking (170 rpm). Optical density at 600 nm (OD_{600}) was measured for each culture for 23 hours.

2.3.14 Nucleotide sequence accession numbers

Nucleotide sequences of the DNA fragment containing *nnh* and the 16S rRNA genes of *Mycobacterium* sp. JS330 were deposited in GenBank under accession numbers HM538831 and HM538832, respectively.

2.4 RESULTS

2.4.1 Isolation and growth of 2NI degrading bacteria

As part of an effort to explore the biodegradation of natural antibiotics, we screened a variety of soil and water samples for bacteria able to utilize 2NI as a carbon and nitrogen source.

Degradation of 2NI accompanied by the accumulation of nitrite was observed in 11 out of 13 enrichments. Isolates able to grow on 2NI or IM2O released nitrite from 2NI. Widespread ability to degrade 2NI indicates a substantial flux of 2NI and its analogs in natural ecosystems and suggests an important ecological role of 2NI, consistent with the widespread presence in soil of actinomycetes and pseudomonads that produce 2NI (32, 39). One isolate designated *Mycobacterium* sp. JS330 grew well on concentrations of 2NI as high as 5 mM. It released ammonia and nitrite, and transiently accumulated IM2O (Fig. 2.1).

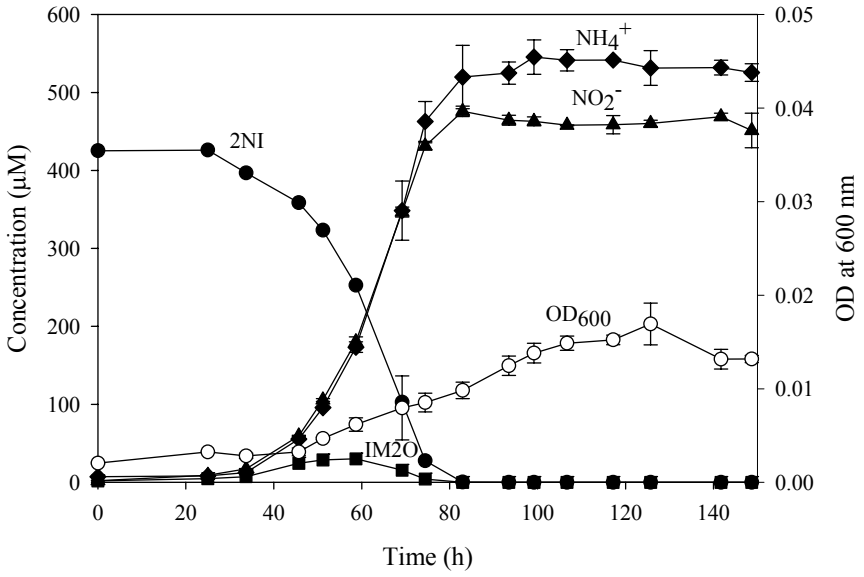

Fig. 2.1 Growth of *Mycobacterium* JS330 on 2NI as the sole carbon, nitrogen and energy source. ●, 2NI; ♦, NH_4^+; ○, OD_{600}; ▲, NO_2^-; ■, IM2O.

The 16S rRNA gene sequence of JS330 was closely (99% identity) related to those of *Mycobacterium brisbanense* and several environmental isolates responsible for degradation of organic compounds. *Mycobacterium brisbanense* strains are ubiquitous in soil, dust, and water. Most are resistant to current anti-tuberculosis agents, and some *M. brisbanense* strains are opportunistic human pathogens (38).

2.4.2 Identification of the gene encoding denitration

Five fosmids from a genomic library of JS330 catalyzed the transformation of 2NI to nitrite and IM2O. One positive clone (pJS900, Table 2.1) was subcloned and a 3.3 kb XbaI/SalI subclone (pJS901, Table 2.1) able to release nitrite and IM2O from 2NI was sequenced. pJS901 carried one complete open reading frame (*nnh*) (Fig. 2.4), which was expressed in pET-21a (Table 2.1). The resulting clone (pJS902, Table 2.1) catalyzed rapid and stoichiometric conversion of 2NI to IM2O and nitrite.

Table 2.1 Bacterial strains, plasmids, and primers used in this study.

Strain, plasmid or primer	Description	Source
Strains		
Mycobacterium sp. JS330	2-Nitroimidazole degrader, isolated from garden soil (Atlanta, Georgia); 16S rRNA gene 99% identical to *Mycobacterium brisbanense* strain NBCS10	This study
Escherichia coli EPI300	Host strain for pJS900	Epicentre (Madison, WI)
NEB 5-alpha *Escherichia coli*	Host strain for pJS901; *E. coli* DH5α derivative	NEB (Ipswich, MA)
E. coli Rosetta 2(DE3)pLysS	Cm^r; host strain for pJS902; contains coding sequences for 7 rare codons	Novagen, (Darmstadt, Germany)
Plasmids		
pCC1FOS	Cm^r; 8.1 kb fosmid vector for the construction of genomic library	Epicentre (Madison, WI)
pUC19	Amp^r; 2686 bp high copy number vector for the double digestion	NEB (Ipswich, MA)
pET21a	Amp^r; 5443 bp over expression vector	Novagen
pJS900	Cm^r; 40 kb pCC1FOS containing *nnh* from JS330	This study
pJS901	Amp^r; 6 kb pUC19 containing *nnh* from JS330; insert DNA cut by XbaI and SalI	This study

TABLE 2.1 CONTINUED

pJS902	Amp^r; 6582 bp pET-21a containing *nnh* from JS330	This study
Primers		
I-341f	5'-CCTACGGGIGGCIGCA-3'	(46)
I-926r	5'-CCGICIATTIITTTIAGTTT-3'	(46)
2NIDenF[a]	5'-CCGCATATGATGACCACTGTTGACAAGAG-3'	This study
2NIDenR[a]	5'-AAAAAGCTTACGCCAAACCCGGTTGCGGA-3'	This study
M13F	5'-TGTAAAACGACGGCCAGT-3'	Genewiz (South Plainfield, NJ)
M13R	5'-CAGGAAACAGCTATGAC-3'	Genewiz (South Plainfield, NJ)
T7	5'-TAATACGACTCACTATAGGG-3'	Genewiz (South Plainfield, NJ)
T7 term	5'-GCTAGTTATTGCTCAGCGG-3'	Genewiz (South Plainfield, NJ)

[a] Engineered NdeI and HindIII recognition sites are underlined for 2NIDenF and 2NIDenR primers, respectively.

2.4.3 Enzyme assays

Extracts prepared from *E. coli* cells carrying pJS901 or pJS902 catalyzed the stoichiometric conversion of 2NI to IM2O and nitrite (Fig. 2.2). The results provide strong evidence that the initial degradation step for 2NI involves denitration to form imidazol-2-one. We further characterized the activity of the enzyme in cell extracts. The optimum pH and temperature were 7.2 and 24°C. Removal of soluble cofactors had no effect on the reaction rates, flavin and iron were not required for the denitration reaction, and addition of electron donors

slightly inhibited the transformation (Table 2.2). EDTA (1 mM), 2-aminoimidazole (0.96 mM), and imidazole (50 mM) had no effect on activity. Oxygen consumption was not detected during enzymatic transformation of 2NI. The results clearly indicated that the enzyme is a hydrolase that does not require metals or other external cofactors.

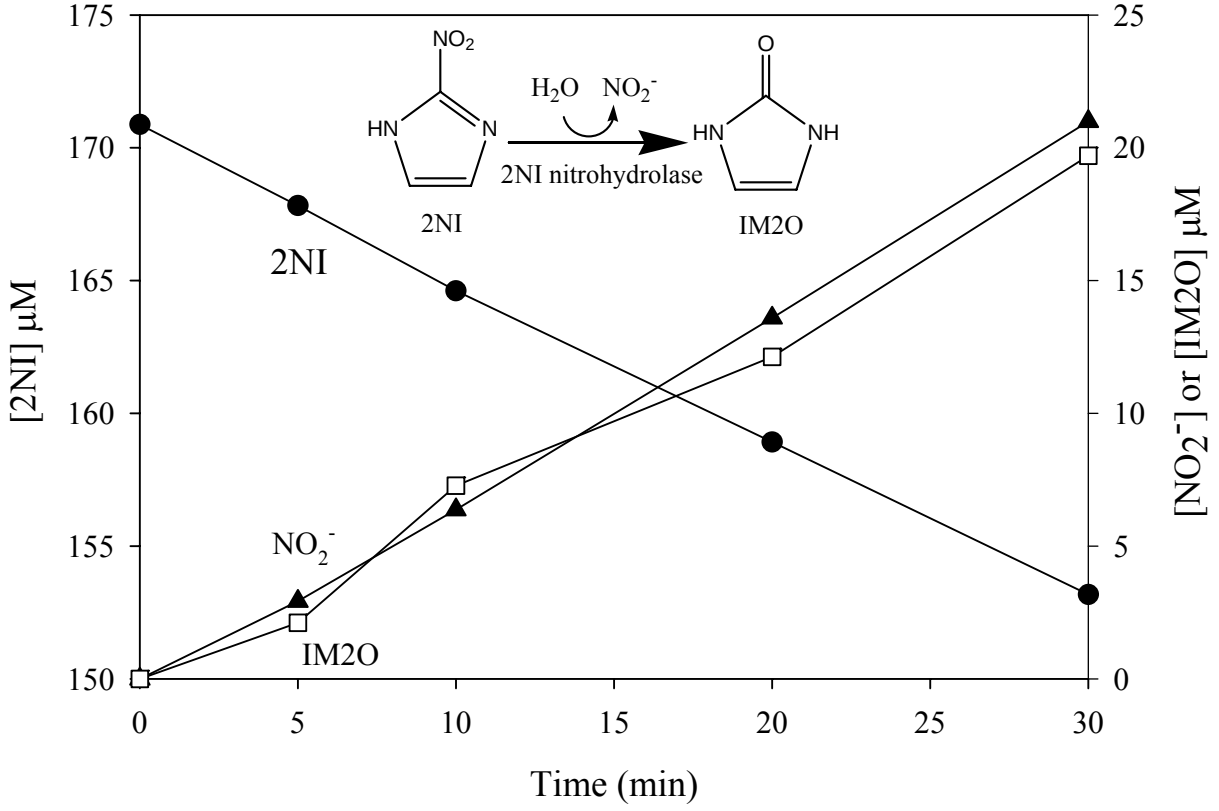

Fig. 2.2 Transformation of 2NI by 2NI nitrohydrolase heterologously expressed in *E. coli.* ●, 2NI; ▲, NO_2^-; □, IM2O. Extract from cells of *E. coli* NEB 5-alpha (pJS901) (Table 2.1) was incubated with 2NI at 30°C. The inset is the proposed initial reaction in the biodegradation of 2-nitroimidazole.

Table 2.2 Effect of additions on denitration of 2NI by ultrafiltered extract from *E. coli* pJS901.

Additions	Relative rate (%)
Fe^{2+}	118 ± 26
Fe^{3+}	115 ± 6.2
NADH	74.7 ± 5
NADPH	71.4 ± 11
FAD	111 ± 12.9
FMN	92.5 ± 6.7
Non-dialyzed	117 ± 17.3
No cofactor	100 ± 6

Reaction mixtures were incubated at 24°C for 5 min. Concentrations of nitrite released were used for the calculation of reaction rates. The experiments were done in duplicate. For the sample with 100% relative rate, the specific activity was 23.6 nmol/min/mg protein. Concentrations were Fe^{2+}, 20 μM; Fe^{3+}, 20 μM; NADPH, 200 μM; NADH, 200 μM; FMN, 20 μM; FAD, 20 μM.

2.4.4 Sequence analysis of *nnh*

We have assigned the *nnh*-encoded enzyme the designation 2-nitroimidazole nitrohydrolase. The amino acid sequence encoded by *nnh* is deeply branched and distantly related to those annotated as *N*-dimethylarginine dimethylaminohydrolase, amidinotransferase, or arginine deiminase (Fig. 2.3) with amino acid identities less than 22%. None of the most closely related sequences have been functionally annotated, but the deduced *nnh* amino acid

sequence contains the conserved domain of *N*-dimethylarginine dimethylaminohydrolase (COG1834) and hydrolytic arginine deiminase (TIGR01078, PRK01388 and COG2235), indicating that they could be related to 2NI nitrohydrolase.

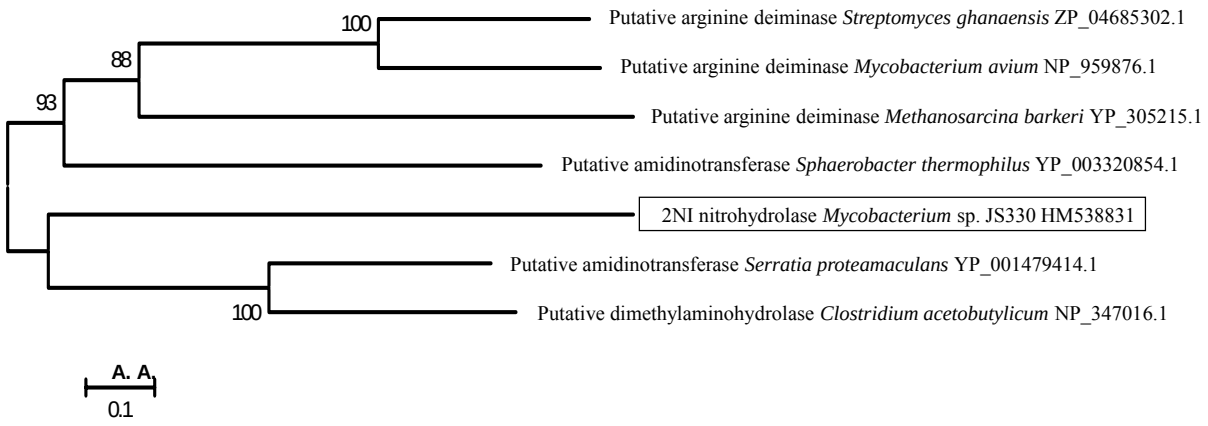

Fig. 2.3 Phylogenetic tree of 2NI nitrohydrolase. The scale bar denotes 0.1 amino acid substitutions per site. Bootstrap analyses were performed on 500 samples, and the percentage greater than 50% is shown at the node. GenBank accession number is indicated at the end of each sequence.

2.4.5 Fosmid sequencing

The DNA sequence of pJS900 contains 18 open reading frames (ORFs) (Fig. 2.4). The open reading frame that encodes 2NI nitrohydrolase (ORF 8, Fig. 2.4) is oriented opposite to the adjacent genes (ORFs 7 and 9) that do not seem to be involved in 2NI degradation. Genes similar to those encoding D-hydantoinase (ORFs 12 & 13, Fig. 2.4) and L-N-carbamoylase (ORF 11, Fig. 2.4) are nearby and likely to be involved in the degradation of IM2O and its intermediates based on analogy to cyclic amidohydrolases (28). Due to the presence of many rare codons in the three ORFs, heterologous expression in *E. coli* seemed unlikely and IM2O accumulated during the transformation of 2NI by pJS900.

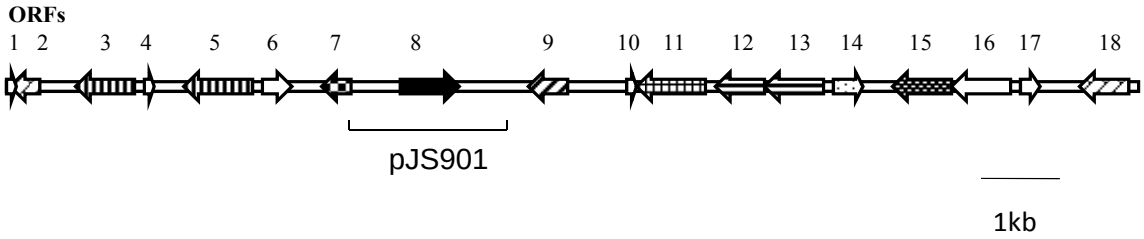

Fig. 2.4 Map of the gene cluster containing 2NI nitrohydrolase. The region subcloned to localize the gene encoding 2NI nitrohydrolase pJS901is indicated below the map. The amino acid identity to the best match from BLASTP search and the corresponding NCBI accession number are shown in parentheses as follows: 1, unknown function; 2, GntR family transcriptional regulator (51%, YP_833070.1); 3, transposase (83%, NP_218155.1); 4, unknown function; 5, transposase (69%, NP_217459.1); 6, unknown function; 7, electron transfer flavoprotein alpha subunit (95%, ZP_03426393.1); 8, amidinotransferase (26%, YP_003320854.1), biochemically characterized as the 2NI nitrohydrolase in this study; 9, pyridoxine biosynthesis protein (87%, ZP_06218322.1); 10, unknown function; 11, L-N-carbamoylase (43%, AAO24769.1); 12, D-hydantoinase (64%, AAO24771.1); 13, D-hydantoinase (58%, AAO24771.1); 14, Rhs family protein (27%, YP_003362093.1); 15, molybdenum cofactor biosynthesis protein (66%, YP_003411862.1); 16, unknown function; 17, unknown function; 18, GntR family transcriptional regulator (43%, ZP_05530754.1).

2.4.6 Antibiotic susceptibility assays

To determine whether *nnh* confers drug resistance to 2NI, it was expressed in *E. coli* NEB 5-alpha, which has been used recently for antibiotic susceptibility assays (15, 41). The results (Fig. 2.5) clearly indicate that heterologous expression of *nnh* confers resistance to 2NI. Furthermore, the ability of *Mycobacterium* sp. JS330 to grow on high concentrations of 2NI (above) is compelling evidence of the ability of the gene to confer drug resistance in *Mycobacterium*.

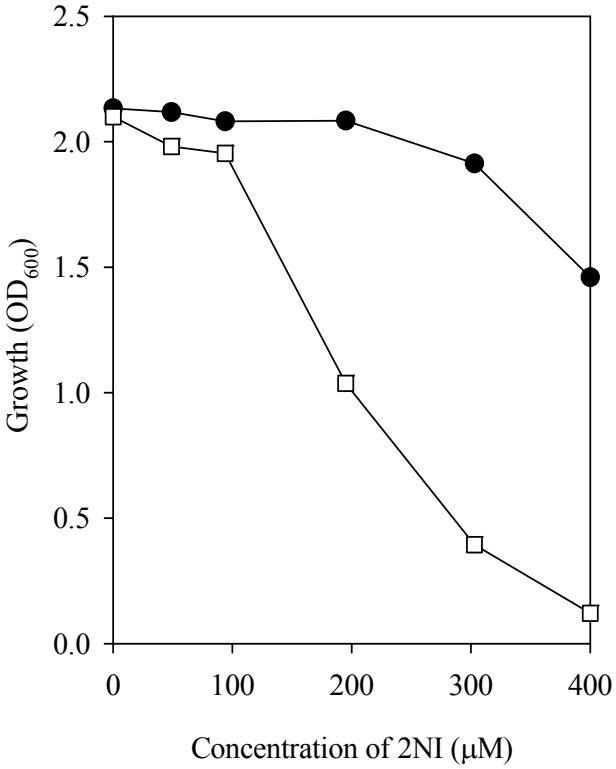

Fig. 2.5 Drug resistance conferred by 2NI nitrohydrolase in *E.coli*. Growth of *E. coli* NEB 5-alpha pJS901 after 23 h (●) (Table 2.1), and *E.coli* NEB 5-alpha pUC19 (vector only) (□).

2.5 DISCUSSION

Biodegradation of synthetic nitroaromatic and nitro aliphatic compounds has been studied extensively (22, 30, 42), but there have been no reports of biodegradation of nitroheterocycles including nitroimidazoles. A substantial amount of previous work has established three mechanisms for the elimination of aromatic and aliphatic nitro groups as nitrite. Monooxygenase and dioxygenase enzymes directly eliminate the nitro groups whereas certain reductases catalyze the formation of non-aromatic hydride-Meisenheimer complexes followed by nitrite release (22, 42). Both types of reactions require electron donors- usually NAD(P)H and the first two require oxygen. Our results indicate that 2NI nitrohydrolase (proposed official name: 2-nitroimidazole

nitrohydrolase) catalyzes a hydrolytic reaction without a requirement for external cofactors, a previously unreported function that belongs in the subclass (E.C. 3.5.99._) of hydrolases acting on carbon-nitrogen bonds but not peptide bonds. 2NI nitrohydrolase activity is stimulated by the addition of NaCl (1 M), which indicates that ionic/proton-donating amino acid(s) may be involved in the activation of water for the hydrolysis (9). Based on analogy with the mechanism of arginine deiminase (24), the Cys residue conserved in 2NI nitrohydrolase and arginine deiminases is likely to be involved in the elimination of the nitro group as nitrite and production of IM2O with the addition of water. The mechanism of the reaction will be determined when the structure of the enzyme is established.

Many natural imidazoles have been reported to have biological activity (1).The biodegradation pathways are known for a few imidazoles such as histidine (36), xanthine (8), imidazole (31), caffeine (12), and 4-aminoimidazole (35). The general rule for the biodegradation pathway of imidazole-type compounds seems to be the formation of a keto group catalyzed by a hydrolase or oxidase to activate the imidazole ring, followed by hydrolase-catalyzed ring cleavage.

Although there is strong evidence that soil bacteria can provide the enzymes responsible for drug resistance in pathogens and a number of soil bacteria able to grow on antibiotics have been isolated (11, 13), the link between drug resistance in pathogens and enzymes in bacteria that biodegrade antibiotics in natural ecosystems is not clear (17, 19). Understanding the environmental reservoirs of resistance is crucial to predicting and preventing the emergence of drug resistance (2, 10).

2NI is representative of a large group of widely used nitroimidazole antibiotics, including metronidazole and the recently developed PA-824. Hydrolytic denitration of 2NI to produce

imidazole-2-one and nitrite prevents the formation of toxic radical, nitroso or hydroxylamino intermediates and thus eliminates the antibiotic effect of 2NI. Elucidating the mechanism and structure of 2NI nitrohydrolase will enable the rational design of new drug candidates that are not substrates for the nitrohydrolase. 2NI nitrohydrolase is a powerful resistance marker because it is insensitive to the presence of oxygen and does not require additional cofactors for activity. In addition, it bypasses the fitness tradeoffs of the common mechanisms of resistance to nitroimidazoles, including altered membrane permeability, defective oxidases resulting in more intracellular oxygen available to remove the unpaired electron from the toxic nitro radical, mutation in the *fgd1* gene blocking the reduction of prodrug PA-824 to toxic products, and the expression of the *nim* genes that reduce 5-nitroimidazoles rapidly to amine derivatives (7, 16). Other bacteria can transform 2NI to the hydroxylamino derivative which is toxic or to the amine derivative which is not (7). In both situations the drugs are transformed but not completely degraded.

Several recent studies have revealed the existence of a reservoir of antibiotic resistance genes in soil bacteria (10-11, 18). Some resistance genes discovered in the microflora from healthy humans (41) or in soil bacteria (15) are distantly related to those from pathogens. The studies did not explore the physiological roles of the enzymes in the soil bacteria and the clinically important nitroimidazoles were not investigated. Although the genomes for many important pathogens including mycobacteria have been sequenced, no homologs of 2NI nitrohydrolase are found in the NCBI database, and the sequence of *nnh* has no similarity to known antibiotic resistance genes.

Our findings reveal a new drug resistance mechanism in soil bacteria prior to its appearance in pathogens, as predicted previously (2, 10-11, 13). Widespread 2NI degrading

bacteria in the environment provide a reservoir for drug resistance genes that could be acquired by pathogens via horizontal gene transfer. Such insight about the evolutionary origins of drug resistance discovered in soil bacteria could provide the basis for minimizing the appearance and spread of resistance in pathogens.

The concentrations of the majority of natural antibiotics in the environment are below the detection limits (2), which is why screening for new antibiotics is done with isolated cultures. Based on our studies with several natural compounds including 2-nitroimidazole, 5-nitroanthranilic acid (33), and 3-nitrotyrosine (22), it seems likely that because naturally occurring organic compounds are biodegradable, the bacteria that degrade them can be detected where the compounds are produced even if the concentrations are negligible. There could be substantial flux of such compounds due to simultaneous production and biodegradation even if the measurable concentrations are very low. If one assumes a spatial link between antibiotic producing and degrading organisms, then screening for antibiotic biodegradation activity by selective enrichment seems to provide a simple and effective way to indicate the presence of sources of the antibiotics.

A major challenge for modern biology resulting from the recent advances in genomics and metagenomics is the massive number of unknown ORFs and genes annotated based solely on weak sequence similarity (29). Functional annotation of genes is needed to narrow the gap between the rapidly growing number of unknown/putative sequences and the dramatically smaller number of biochemically characterized enzymes. It is difficult or impossible to assign function to unknown genes based exclusively on sequence comparisons. In contrast, the discovery of new enzymes, their physiological roles, and then the genes that encode them ensures correct functional annotations (15, 20, 45). Based on recent work (30, 33, 45) including

this study, it seems that biodegradation pathways for the millions of natural organic compounds (14) could explain the functions of many of the genes encoding novel enzymes.

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CHAPTER 3

Biodegradation of 5-Nitroanthranilic Acid by *Bradyrhizobium* sp. Strain JS329

3.1 ABSTRACT

Biodegradation of synthetic compounds has been studied extensively, but the metabolic diversity required for catabolism of many natural compounds has not been addressed. 5-Nitroanthranilic acid (5NAA), produced in soil by *Streptomyces scabies*, is also the starting material for synthetic dyes and other nitroaromatic compounds. *Bradyrhizobium* JS329 was isolated from soil by selective enrichment with 5NAA. When grown on 5NAA the isolate released stoichiometric amounts of nitrite and half of the stoichiometric amounts of ammonia. Enzyme assays indicate that the initial step in 5NAA degradation is an unusual hydrolytic deamination to form 5-nitrosalicylic acid (5NSA). Cloning and heterologous expression revealed the genes that encode 5NAA deaminase (*naaA*) and 5NSA dioxygenase (*naaB*) that cleaves the aromatic ring of 5NSA without prior removal of the nitro group. The results provide the first clear evidence for the initial steps in biodegradation of amino-nitroaromatic compounds and reveal a novel

deamination reaction for aromatic amines.

3.2 INTRODUCTION

The research on biodegradation/ biotransformation of nitro compounds has focused on synthetic chemicals, but there are a substantial number of natural nitro substituted compounds whose metabolism has not been explored. The biodegradation pathways for natural nitro compounds probably provided the metabolic diversity that enabled the rapid and recent evolution of pathways for degradation of synthetic nitro compounds.

5-Nitroanthranilic acid (5NAA), a natural nitroaromatic compound, is produced by *Streptomyces scabies*, but its physiological role is unclear (15). Synthetic 5NAA is used as the starting material for various nitroaromatic compounds and dyes (3). Substitution of the aromatic ring with amino, nitro and carboxyl functional groups creates an interesting challenge for catabolic enzymes because any of the three groups could serve as a point of attack for dioxygenase enzymes prior to ring cleavage.

Synthetic nitroanilines are toxic and used for the synthesis of pharmaceuticals, dyes and pigments (27). In sewage, nitroanilines can be formed from the corresponding dinitroaromatic compounds under aerobic or anaerobic conditions (11). Early reports indicated that nitroanilines were resistant to biodegradation (1, 9, 11), but 4-nitroaniline was degraded by *Pseudomonas* sp. strain P6 (32) and *Stenotrophomonas* strain HPC 135 (26). Saupe reported that 3-nitroaniline could be degraded aerobically (27). The

biodegradation pathways of nitroanilines are unknown, and they are typically classified as nondegradable or poorly degradable compounds (27).

As part of a search for novel metabolic diversity and an effort to study the degradation pathway for recalcitrant nitroanilines, we report here the biodegradation of 5NAA as the sole carbon, nitrogen and energy source by *Bradyrhizobium* sp. JS329. The degradation pathway involves an unusual hydrolytic removal of the amino group and subsequent ring fission without prior removal of the nitro group.

3.3 MATERIALS AND METHODS

3.3.1 Isolation and growth of 5NAA degrading bacteria

Soils were suspended (10%, wt/vol) in nitrogen-free minimal medium (BLK, pH 7.2) (4) containing 5NAA (50 μM) and incubated at 30°C with shaking. When 5NAA disappeared subcultures were transferred to fresh medium with increasing concentrations of 5NAA. After repeated subculture into media with increasing levels of 5-NAA, a pure culture was isolated on agar plates containing BLK and 5NAA (500 μM).

Isolates were routinely grown on BLK agar plates or in BLK liquid medium supplemented with 5NAA (500 μM). When large amounts of cells were required, cultures were grown in BLK supplemented with succinate (7.5 mM) and 5NAA (500 μM) as the carbon and nitrogen sources. The non-induced cultures were grown with succinate (7.5 mM) and NH_4Cl (500 μM). Exponential-phase cells were harvested by centrifugation and

washed twice with phosphate buffer (20 mM, pH 7.0) prior to use.

3.3.2 Analytical methods

5NAA, 5-nitrosalicylic acid (5NSA), gentisate and salicylate were analyzed by high performance liquid chromatography (HPLC) with an Agilent Eclipse XDB-C_{18} column (4.6 mm by 150 mm; 5 μm), using the method described previously (21). 5NAA was monitored at 370 nm (RT- 7.1 min), 5NSA was detected at 305 nm (RT- 7.5 min), gentisate was monitored at 330 nm (RT- 5.4 min), and salicylate was detected at 305 nm (RT- 7.3 min).

Ammonia and nitrite were measured as reported previously (21). Protein was measured with a Pierce (Rockford, IL) BCA Protein Assay Reagent Kit.

3.3.3 Chemicals

5-Nitroanthranilic acid, 4-nitrocatechol, salicylic acid, and gentisic acid were from Sigma-Aldrich (Milwaukee, WI). 5-Nitrosalicylic acid was from Eastman Kodak (Rochester, NY). 5-Hydroxyanthranilic acid was from Acros Organics (Geel, Belgium).

3.3.4 Identification of bacteria

A Genomic DNA Purification System (Promega, Madison, WI) was used to extract

genomic DNA, which was the template for 16S rDNA amplification by PCR with 8F (5'-AGAGTTTGATCCTGGCTCAG-3') (18) and 1489R (5'-TACCTTGTTACGACTTCA-3') (30). The 16S rRNA gene sequence (794 bp) was compared to published DNA sequences deposited in GenBank (http://www.ncbi.nlm.nih.gov/) using BLASTN (2).

3.3.5 Respirometry

Oxygen uptake was measured polarographically at 25°C with a Clark-type oxygen electrode connected to a YSI model 5300 biological oxygen monitor.

3.3.6 Cell extracts and enzyme assays

Cells were passed three times through a French pressure cell (20,000 psi). The cell lysate was clarified by centrifugation (20,000 x g, 4°C, 20 min). The supernatant was concentrated by ultrafiltration (Microcon Centrifugal Filter Devices, Molecular weight cut-off: 10 kDa) at 4°C, washed twice with cold phosphate buffer, then the rententate was suspended in cold phosphate buffer. The 5NAA deaminase and 5NSA 1,2-dioxygenase enzyme assays were performed at 30°C in potassium phosphate buffer (pH 7.0, 20 mM). Typical assays contained 0.6-3.6 mg protein and 0.05-0.5 μmol substrate in a total volume of 1 ml. Trifluoroacetic acid (TFA) was added to the mixture (1:100 vol/vol) to stop the reaction. The acidified mixture was clarified by centrifugation before HPLC

analysis.

3.3.7 Gene library construction and screening

Genomic DNA (3 μg) from 5NAA-grown *Bradyrhizobium* JS329 cells was extracted with an UltraClean Microbial DNA Isolation kit (Carlsbad, CA). DNA was randomly sheared by vortexing for 2 min. The sheared DNA fragments (approximately 30 kb in size) were end-repaired to generate blunt, 5'-phosphorylated ends. The blunt end fragments were ligated into CopyControl Vector pCC1FOS, and transfected into phage T1-resistant *E. coli* strain Epi300-$T1^R$ as described in the manufacturer's protocol (Epicentre Biotechnologies, Madison, WI) to create a genomic library.

Clones were screened for 5NAA-degrading activity after 40 hours of growth in 96-well plates containing LB medium supplemented with chloramphenicol (12.5 μg/ml), 1x Fosmid CopyControl Induction Solution (Epicentre Biotechnologies, Madison, WI), and 5NAA (100 μM). Because 5NAA is yellow, a clone designated pJS800 able to catalyze 5NAA transformation was detected by loss of the yellow color (Table 3.1).

Table 3.1 Bacterial strains and plasmids used in this study

Strain or plasmid	Description	Source
Strains		
Bradyrhizobium sp. strain JS329	5-Nitroanthranilic acid degrader	This study
Escherichia coli EPI300	Host strain for pJS800, pJS801, and pJS802	Epicentre (Madison, WI)
Plasmids		
pCC1FOS	Cm^r, 8.1 kb fosmid vector for the construction of genomic library	Epicentre (Madison, WI)
pJS800	Cm^r, 40 kb pCC1FOS containing *naaA* and *naaB* from JS329 ($5NAA^+$ $5NSA^+$)	This study
pJS801	Cm^r Km^r, transposon Tn5 insertion into *naaA* of pJS800 ($5NAA^-$ $5NSA^+$)	This study
pJS802	Cm^r Km^r, transposon Tn5 insertion into *naaB* of pJS800 ($5NAA^+$ $5NSA^-$)	This study

3.3.8 Identification of key genes

Fosmid pJS800 was purified from the *E. coli* host with a FosmidMAX DNA Purification Kit (Epicentre Biotechnologies, WI) and subjected to mutagenesis in vitro using an Ez-Tn5 <KAN-2> insertion kit (Epicentre Biotechnologies, Madison, WI). The randomly mutated fosmids were reintroduced into *E. coli* (TranforMax EC100, Epicentre Biotechnologies, Madison, WI) by electroporation. Tn5-transposon mutants were selected on LB agar plates supplemented with chloramphenicol (12.5 μg/ml) and kanamycin (50 μg/ml). The resistant mutants were screened for the ability to transform 5NAA or 5NSA

by HPLC. Clones pJS801 and pJS802 (Table 3.1) were sequenced to confirm the transposon insertion site.

The mutants that lost the ability to transform 5NAA or 5NSA were sequenced by primer walking, starting with Ez-Tn5 <KAN-2> specific outward reading primers (Ez-Tn5 <KAN-2> FP-1 forward primer and Ez-Tn5 <KAN-2> RP-1 reverse primer, Epicentre Biotechnologies, Madison, WI). DNA was sequenced by Nevada Genomics Center (Reno, NV). Sequence pairwise-comparison was performed against the GenBank using BLAST. The promoter was predicted using the Promoter Prediction tool on the Berkeley Drosophila Genome Project website (http://www.fruitfly.org/seq_tools/promoter.html).

3.3.9 Nucleotide sequence accession numbers

Nucleotide sequences of the 16S rRNA genes of *Bradyrhizobium* JS329 and the sequences of genes involved in 5NAA degradation were deposited in GenBank under accession numbers GU188568 and GU188569, respectively.

3.4 RESULTS

3.4.1 Isolation and identification of 5NAA degrading bacteria

Biodegradation of 5NAA was detected in enrichment culture after 8 days with 5NAA as the sole source of carbon, nitrogen, and energy. Among several soil samples only soil

from a potato farm yielded a bacterial isolate able to grow on 5NAA. The partial 16S rDNA sequence (794 bp) of the isolate was 99% identical to the 16S rDNA of *Bradyrhizobium elkanii*, but because 16S rDNA does not discriminate among *Bradyrhizobium* species (31), the isolate was designated *Bradyrhizobium* sp. JS329.

3.4.2 Growth of bacteria

JS329 grew best at pH 7.2 and 30°C on concentrations of 5NAA up to 5 mM. During growth on 5NAA, JS329 released stoichiometric amounts of nitrite along with some ammonia, which indicates that nitrite is released without prior reduction or assimilation and that the ammonia serves as the source of nitrogen. 5-Nitrosalicylic acid (5NSA) accumulated transiently during 5NAA degradation (Fig. 3.1). The release of 5NSA suggested that the amino group was released before the nitro group.

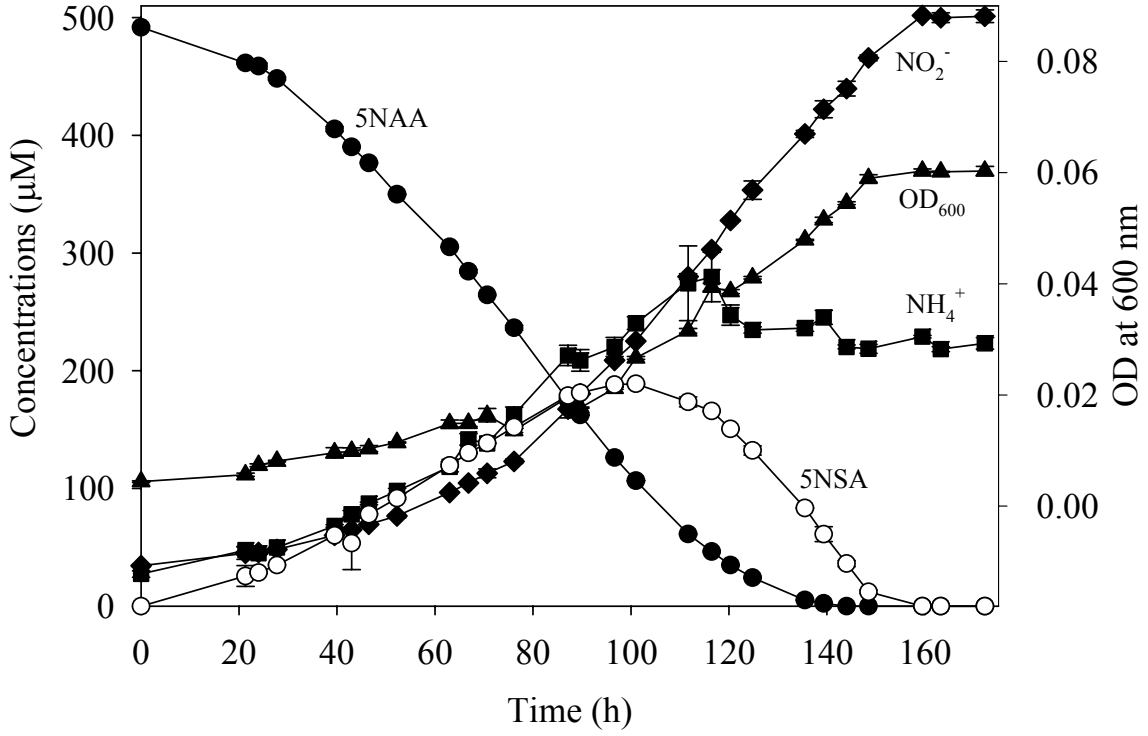

Fig. 3.1 Growth of *Bradyrhizobium* sp. JS329 on 5NAA as the sole carbon, nitrogen and energy source. ●, 5NAA; ♦, NO_2^-; ○, 5NSA; ▲, OD_{600}; ■, NH_4^+.

JS329 also grew on 5NSA as the sole carbon, nitrogen and energy source with the accumulation of nitrite (0.5 mol NO_2^-/mol 5NSA) in the culture medium. We hypothesized that the initial attack might be on the adjacent amino and carboxyl groups similar to the initial step in the anthranilic acid degradation pathway to yield 4-nitrocatechol, but 4-nitrocatechol did not serve as a growth substrate, which suggested that 4-nitrocatechol is not involved in the degradation of 5NAA. The growth yields were similar for growth on 5NAA and 5NSA (data not shown). Either ammonium or nitrite could serve as nitrogen source for growth. JS329 grew well with 5NAA as the nitrogen

source and glucose, succinate, pyruvate, or acetate as the carbon source.

3.4.3 Respirometry

The above preliminary experiments suggested that 5NSA was produced from 5NAA and that nitrite was formed by subsequent removal of nitro group. Possible transformation pathways of 5NAA could also involve the formation of 4-nitrocatechol (17), or 5-hydroxyanthranilic acid along with release of nitrite (5) (Fig. 3.2). The three potential intermediates were tested for the ability to stimulate oxygen uptake by 5NAA or succinate grown cells (Table 3.2). Negative results with 4-nitrocatechol and 5-hydroxyanthranilic acid suggested that the two compounds are not involved in the 5NAA degradation pathway. Stimulation of oxygen uptake by 5NAA, and 5NSA in 5NAA-grown cells, but not in succinate-grown cells, suggested that inducible enzymes catalyzing transformation of 5NAA, and 5NSA were involved in the pathway.

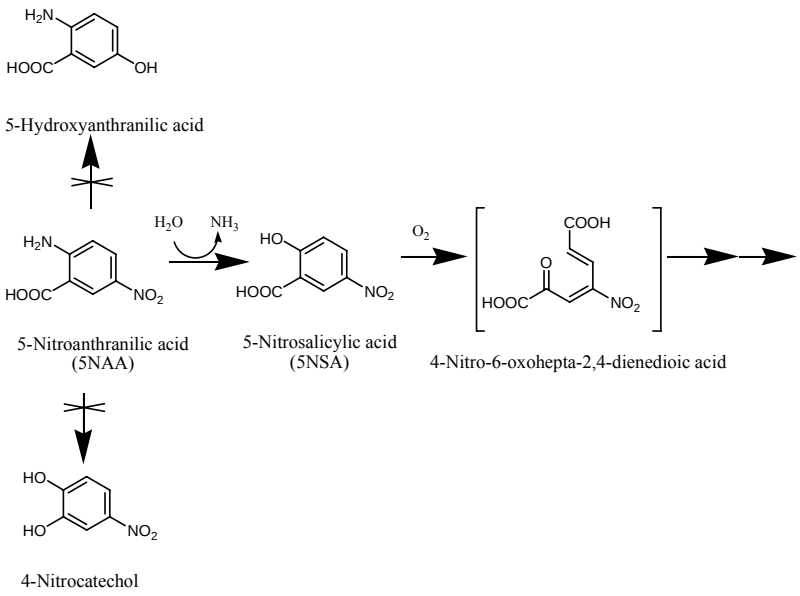

Fig. 3.2 Hypothetical initial reactions in the biodegradation of 5NAA. The middle pathway was supported by this study.

Table 3.2 O_2 uptake by 5NAA or succinate grown cells

Test substrate	Oxygen uptake (nmol O_2/min/mg of protein) by cells grown in	
	5-Nitroanthranilic acid	Succinic acid
5-Nitroanthranilic acid[a]	5.7 ± 0.42	ND
5-Nitrosalicylic acid[b]	6.6 ± 0.85	ND
Gentisic acid	2 ± 0.84	ND
5-Hydroxyanthranilic acid	ND	ND
4-Nitrocatechol	ND	ND
Succinic acid	NT	5.4

Reaction mixtures contained 1.8 ml of oxygen-saturated phosphate buffer (0.02 M; pH 7.0; 25°C), cells, and test substrates (20 μM). Experiments were done in duplicate except for succinate control. ND: Not detected; NT: Not tested.

[a] The stoichiometry was 1.9 ± 0.1 mol O_2/ mol substrate

[b] The stoichiometry was 1. 7 ± 0.1 mol O_2/ mol substrate

3.4.4 Cloning of the genes involved in 5NAA degradation

When the fosmid library was screened for clones with the ability to transform 5NAA, three out of 5000 clones were able to transform both 5NAA and 5NSA to non UV absorbing metabolites. Tn5 mutants were prepared from pJS800. One group of mutants exemplified by pJS801 lost the ability to transform 5NAA, but not 5NSA. Mutants (pJS802) that lost the ability to transform 5NSA transformed 5NAA stoichiometrically to 5NSA (data not shown). Primer walking yielded about 2.2 kb of sequence containing two adjacent and divergently transcribed genes (Fig. 3.3). The 5NAA deaminase (encoded by *naaA* in Fig. 3.3) is distantly (34% amino acid identity) related to an uncharacterized M20/M25/M40 family peptidase from *Hyphomonas neptunium* ATCC 15444. 5NSA 1,2-dioxygenase (encoded by *naaB* in Fig. 3.3) is distantly (29% amino acid identity) related to gentisate 1,2-dioxygenase from *Oligotropha carboxidovorans* OM5 (23) and salicylate 1,2-dioxygenase (13) from *Pseudaminobacter salicylatoxidans* (22% amino acid identity). The deduced 5NSA 1,2-dioxygenase amino acid sequence contained a conserved domain in common with salicylate dioxygenase and gentisate 1,2-dioxygenase (TIGR02272, and COG3435) (19). The promoter sequence for the *naaB* gene, found in the 400 bp intergenic region, is TCGTTCTGTAGAACGAACGACGATCTGTATATTGTTACTG**G**GAGGGTGAC, and the promoter sequence for the *naaA* gene is TGAATTTTGGCTGTTGACCGTAGACAG AACATCGTTTAAT**A**CCCCGAACA. The transcription starts at G or A (underlined bold

character). The results for 5NAA and 5NSA transformation by the clones are consistent with hydrolytic deamination of 5NAA to 5NSA followed by ring-cleavage catalyzed by the dioxygenase.

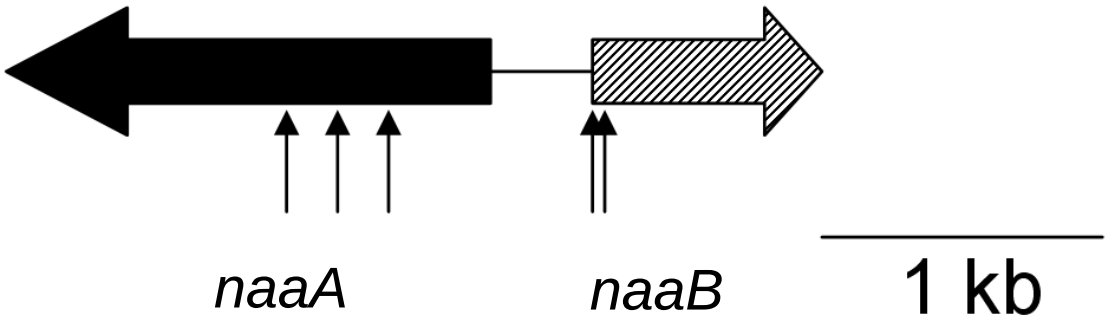

Fig. 3.3 Identification of key genes. *naaA*: gene encoding 5NAA deaminase; *naaB*: gene encoding 5NSA 1,2-dioxygenase. Sites of Tn5 transposon insertion are marked by arrows.

3.4.5 Enzyme assays

Ultrafiltered extracts from 5NAA grown cells of *Bradyrhizobium* JS329 catalyzed stoichiometric transformation of 5NAA to 5NSA (specific activity 0.8 ± 0.32 nmol/min/mg protein). No added cofactors were required for the transformation (Fig. 3.4). The results strongly suggest that the initial reaction involves hydrolytic elimination of the amino group. The equal stoichiometry of oxygen consumption versus substrate for 5NAA and 5NSA in intact cells clearly shows that no oxygen is involved in the conversion of 5NAA to 5NSA and strongly supports the above conclusions (Table 3.2).

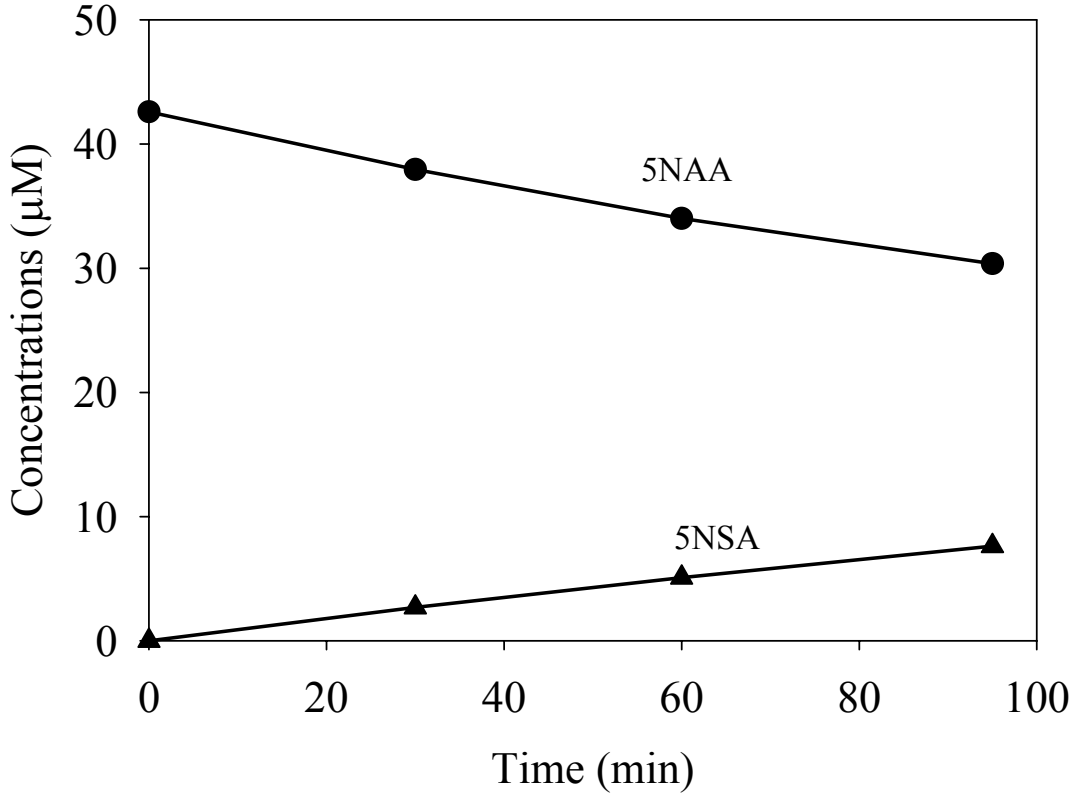

Fig. 3.4 Stoichiometric accumulation of 5-nitrosalicylate (5NSA) from 5NAA when ultrafiltered extract from 5NAA-grown cells was incubated with 5NAA at 30°C. ●, 5NAA; ▲, 5NSA.

Enzymes in crude extracts of 5NAA grown cells of *Bradyrhizobium* JS329 catalyzed the oxidation of 5NSA (specific activity 1.2 ± 0.2 nmol/min/mg protein). Gentisate was also oxidized at rates (19 ± 1.7 nmol/min/mg protein) higher than in intact cells which suggests that transport was limiting for gentisate. However, enzymes in cell extracts of the *E. coli* containing fosmid clone pJS800 harboring *naaB* did not attack gentisate, but did transform salicylate (specific activity 3.9 ± 0.9 nmol/min/mg protein) and 5NSA (specific activity 11 ± 0.5 nmol/min/mg protein). The results indicate that gentisate dioxygenation activity in JS329 was not catalyzed by the enzyme transforming 5NSA,

but the expression of gentisate dioxygenase gene(s) in another locus was induced during growth on 5NAA.

Table 3.3 Effect of additions on transformation of 5NSA by ultrafiltered extract from 5NAA-grown cells.

Additions	Relative rate (%)
Fe^{2+}	100
NADH	23.3 ± 1.7
Fe^{3+}	9.4 ± 1.2
Fe^{2+}, NADH	87.7 ± 2.2
Fe^{2+}, NADH, FAD	102.1 ± 0.6
Fe^{2+}, NADH, FMN	90.4 ± 0.8
Fe^{2+}, NADH, DTT	70 ± 14
No cofactor	17 ± 2.4

Cells were incubated at 30°C with shaking. Concentrations were Fe^{2+}, 50 μM; Fe^{3+}, 50 μM; NADH, 150 μM; FMN, 10 μM; FAD, 10 μM; DTT, 1 mM.

Ultrafiltration abolished the enzymatic activity against 5NSA, which was restored by addition of ferrous but not ferric ions. Addition of flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN), dithiothreitol (DTT), or NAD(P)H had no effect on the activity (Table 3.3). Nitrite was not detected during the transformation of 5NSA, and no UV absorbing metabolites were detected by HPLC.

5NSA oxidation catalyzed by cell extracts required 0.97 ± 0.09 mol O_2/mol 5NSA. Upon incubation with 2,2'-dipyridyl (1 mM) or *o*-phenanthroline (1 mM), the enzymatic activity was inhibited by 97.7% or 100%, respectively. The requirement for oxygen and

Fe^{2+} but no external electron donor suggested that 5NSA transformation is catalyzed by a ring-cleavage dioxygenase.

The properties of the enzyme catalyzing 5NSA oxidation were similar to those of salicylate 1,2-dioxygenase: 1 mol of oxygen consumed per mol of substrate; 40%-50% of original activity lost after 1 week of storage at -20°C; Fe^{2+} required; NAD(P)H not required; active against salicylate; and inhibited by iron chelators (12).

3.5 DISCUSSION

Our study provides the first clear evidence for the initial steps in biodegradation of nitroanilines. The enzymatic removal of either the amino group or the nitro group might be expected to initiate biodegradation of such molecules. Amino groups attached to aromatic rings are usually removed in reactions catalyzed by dioxygenases. For example, aniline is converted to catechol by aniline dioxygenase, a multi-component dioxygenase (10, 29). Anthranilate (2-aminobenzoate) is transformed to catechol through the simultaneous removal of the adjacent amino and carboxyl groups by a dioxygenase in *Pseudomonas* (17) or *Burkholderia cepacia* DBO1 (6). A flavoprotein hydroxylase in the yeast *Trichosporon cutaneum* (24) converts anthranilate into 2,3-dihydroxybenzoate, whereas enzymes in *Nocardia opaca* convert it to 5-hydroxyanthranilate (5). The different deamination mechanisms among 5NAA-, anthranilate-, and aniline-degrading enzymes might be caused by steric or electronic effects of the nitro group in the 5NAA molecule. Aminohydrolases are commonly involved in elimination of amino groups from

aliphatic and heterocyclic compounds (16, 28) but have not been reported to act on aromatic compounds. Purification and characterization of the novel enzyme will be required to determine substrate range and the mechanism of the unusual reaction.

The fact that gentisate can stimulate the oxygen uptake by 5NAA grown cells (Table 3.2) at first suggested that 5NSA was converted to gentisate by a flavoprotein analogous to nitrophenol monooxygenase (14). 5NSA transformation in cell extracts without an added electron donor and no nitrite accumulation established clearly that another mechanism must be involved. Our results indicate that the reaction is a ring-cleavage catalyzed by a dioxygenase that is active against both 5NSA and salicylate, but not gentisate. The finding is consistent with the report that an enzyme from *Pseudaminobacter salicylatoxidans* with 28% identity to gentisate 1,2-dioxygenase from *Pseudomonas alcaligenes* NCIMB 9867 can oxidize salicylate and a variety of substituted salicylates, but not 5NSA (13). Our results indicate that the ring-cleavage dioxygenase has evolved the ability to oxidize 5NSA specifically.

The conservation of a histidine pair is essential for the anchoring of ferrous ion in the catalytic center of salicylate and gentisate 1,2-dioxygenases (7, 13). Alignment of the sequences of salicylate 1,2-dioxygenase from *Pseudaminobacter salicylatoxidans*, gentisate 1,2-dioxygenase from *Burkholderia multivorans* CGD2M, gentisate 1,2-dioxygenase from *Oligotropha carboxidovorans* OM5, 1-hydroxy-2-naphthoic acid dioxygenase from *Mycobacterium* sp. CH1, and 5NSA 1,2-dioxygenase reveals that the histidine pair is highly conserved (Fig. 3.5).

Fig. 3.5 Multiple sequence alignment of 5NSA 1,2-dioxygenase from *Bradyrhizobium* JS329, salicylate-1,2-dioxygenase from *Pseudaminobacter salicylatoxidans* BN12 (AY323951.1), 1-hydroxy-2-naphthoic acid dioxygenase from *Mycobacterium sp.* CH1 (ACN38281.1), gentisate 1,2-dioxygenase from *Burkholderia multivorans* CGD2M (ZP_03569724.1) and gentisate 1,2-dioxygenase from *Oligotropha carboxidovorans* OM5 (YP_002287389.1). The conserved histidine pair is present at positions 123 and 125. The alignment in the two ends was trimmed due to the absence of conserved sites. SA: salicylate; GA: gentisate; 5NSA: 5-nitrosalicylic acid; HNA: 1-hydroxy-2-naphthoic acid.

Inducible activities of 5NAA deaminase and 5NSA 1,2-dioxygenase were detected in both intact cells and cell extracts at specific activities sufficient to support the growth of JS329. The results indicate that the pathway is initiated by a novel hydrolytic deaminase to produce 5NSA, which in turn is oxidized by a ring-fission dioxygenase (Fig. 3.2). The mechanism of removal of the nitro group is a particularly interesting question.

For most nitrophenols nitro group removal is catalyzed by NAD(P)H-dependent monooxygenase enzymes prior to cleavage of the aromatic ring (22). Biodegradation of 5NAA, 2,6-DNT and picric acid are exceptions to the general rule (8, 20). In such cases the nitrophenol serves as the ring-fission substrate. The mechanism of release of the nitro group from the ring-fission product is unknown (20).

The failure to detect 5NAA degrading bacteria in the majority of the soil samples suggests that the distribution of 5NAA degraders is limited as did the earlier work by Hallas & Alexander (11). It seems likely that ecosystems that harbor 5NAA producers would also harbor 5NAA degraders, but the relationship between 5NAA production and degradatioin remains to be determined.

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Chapter 4

Molecular and biochemical characterization of the 5-nitroanthranilic acid degradation pathway in a *Bradyrhizobium*

4.1 ABSTRACT

Biodegradation pathways of synthetic nitroaromatic compounds and anilines are well documented, but little is known about those of nitroanilines. We previously reported that the initial step in 5-nitroanthranilic acid (5NAA) degradation by *Bradyrhizobium* JS329 is a hydrolytic deamination to form 5-nitrosalicylic acid (5NSA), followed by ring fission catalyzed by 5NSA dioxygenase. The mechanism of release of the nitro group was unknown. In this study we subcloned sequenced and expressed 5NAA deaminase (5NAA aminohydrolase, NaaA), 5NSA dioxygenase (NaaB) and lactonase (NaaC), the key genes responsible for 5NAA degradation. Sequence analysis revealed that NaaA is a hydrolytic metalloenzyme with a narrow substrate range. The nitro group is spontaneously eliminated as nitrite concomitant with formation of lactones from the ring-fission product of 5NSA dioxygenation. The elimination of the nitro group during lactone formation is a previously unreported mechanism for denitration of nitro aliphatic compounds.

4.2 INTRODUCTION

Nitro-substituted compounds are widely used as dyes, pesticides, synthetic intermediates, and explosives (29, 40). The research on biodegradation of nitro compounds has focused on synthetic chemicals and a great deal is known about the biochemistry and molecular biology of the pathways. The degradation pathways for synthetic compounds appear to have evolved by recruitment and assembly of genes from other pathways in response to the recent introduction of such chemicals into the biosphere (6, 18, 44, 46). Over 200 natural nitro compounds (Nishino, S. F., J. C. Spain, and R. J. Parry, submitted for publication) are produced by a variety of microbes, plants, and animals (5, 20), but little is known about their biodegradation mechanisms.

The presence of amino and nitro groups in aromatic compounds presents microorganisms with a more complex challenge than the presence of either group alone. We recently reported the biodegradation of the natural aminonitroaromatic compound 5-nitroanthranilic acid (5NAA) (31) produced by *Streptomyces scabies*, the causative agent of potato scab (22). In *Bradyrhizobium* JS329 isolated from potato farm soil the biodegradation of 5NAA was initiated by a deamination reaction catalyzed by a novel aminohydrolase (5NAA deaminase, NaaA) to produce 5-nitrosalicylic acid (5NSA), followed by an unusual ring-fission dioxygenation (31). The genes involved were localized on a 40 kb fosmid clone (31). The properties of 5NAA deaminase (NaaA) and the mechanism of removal of the nitro group were not established.

The mechanisms of removal of the nitro group from the ring-fission products of picric acid and 2,6-dinitrotoluene (2,6-DNT) are long-standing mysteries (9, 15, 27). The degradation of 2,4,6-trinitrophenol (picric acid) by *Nocardioides simplex* FJ2-1A is

initiated by the reduction of the aromatic ring and subsequent release of nitrite, catalyzed by F420 dependent enzyme system, followed by hydrolytic ring fission to produce 4,6-dinitrohexanoate (4,6-DNH). The mechanism by which the nitro group is subsequently removed from the aliphatic ring fission product is unknown (9). Similarly, biodegradation of 2,6-DNT is initiated by a dioxygenase to form 3-methyl-4-nitrocatechol (3M4NC) accompanied by the release of one nitro group as nitrite. The nitro group of 3M4NC is removed by an unknown mechanism after ring cleavage (27).

In this study we characterized 5NAA deaminase and elucidated the mechanism of elimination of the nitro group from the ring-fission product. Transition divalent metals are essential for the hydrolytic activity of 5NAA deaminase, and the nitro group is spontaneously removed as nitrite during the lactonization of the ring-fission product. The enzymes and mechanisms involved in the biodegradation of 5NAA are unusual and distinct from those previously described for oxygenase-catalyzed removal of phenyl nitro and amino groups.

4.3 MATERIALS AND METHODS

4.3.1 Fosmid DNA sequencing and *in silico* analysis

Fosmid DNA pJS800 (Table 4.1) was purified with the FosmidMAX DNA Purification Kit (Epicentre Biotechnologies, WI), and sent to the Emory GRA Genomic Center for 454 pyrosequencing. The reads were assembled with CLC Genomics Workbench. Open reading frames (ORFs) were identified by GeneMark (3) (http://exon.biology.gatech.edu/), then compared to the GenBank database by BLASTP (1).

Table 4. 1 Bacterial strains, plasmids, and primers used in this study

Strains, plasmids, or primers	Description	Source
Strains		
Bradyrhizobium sp. strain JS329	5-Nitroanthranilic acid degrader	(31)
Escherichia coli EPI300	Host strain for pJS800, and Tn5 transposon mutants	Epicentre (Madison, WI)
E. coli Rosetta 2(DE3)pLysS	Cm^r, Host strain for pJS803, pJS804, pJS805, contains coding sequences for 7 rare codons	Novagen
Plasmids		
pCC1FOS	Cm^r, 8.1 kb fosmid vector for the construction of genomic library	Epicentre (Madison, WI)
pET21a	Amp^r, 5443 bp, over expression vector	Novagen
pJS800	Cm^r, 40 kb pCC1FOS containing *naaA* and *naaB* from JS329 ($5NAA^+$ $5NSA^+$)	(31)
pJS803	Amp^r, 6720 bp pET-21a containing the gene encoding 5NAA deaminase (NaaA) from *Bradyrhizobium* JS329	This study

Table 4.1 continued

pJS804	Amp^r, 6066 bp pET-21a containing the gene encoding 5NSA dioxygenase (NaaB) from *Bradyrhizobium* JS329	This study
pJS805	Amp^r, 6190 bp pET-21a containing the gene encoding lactonase (NaaC) from *Bradyrhizobium* JS329	This study
Primers		
naaAF[a]	5'- AAAGGATCCATGGCTGGAAGTAACGACGT -3'	This study
naaAR[b]	5'- AAACTCGAGGGGCGTACGATTGCACAGAT -3'	This study
naaBF[a]	5'-AAAGGATCCATGAAATGGAGCAACAAAGA-3'	This study
naaBR[b]	5'-AAACTCGAGTTATTCGCCTTGCTTGAGAA-3'	This study
naaCF[a]	5'-AAAGGATCCATGGCAACTGAAACCATCGC-3'	This study
naaCR[b]	5'-AAACTCGAGTCACACCGTGCGCTTGC-3'	This study

[a] Engineered BamHI recognition sites are underlined for forward primers.

[b] Engineered XhoI recognition sites are underlined for reverse primers.

4.3.2 Transposon mutagenesis and screening

The fosmid clone pJS800 (31) able to release nitrite from both 5NAA and 5NSA was subjected to Tn5 transposon mutagenesis with the Ez-Tn5 <KAN-2> insertion kit (Epicentre Biotechnologies, Madison, WI) according to the manufacturer's protocol. Colonies able to grow on LB agar supplemented with chloramphenicol (12.5 μg/ml) and kanamycin (50 μg/ml) were transferred to 96-well plates containing LB liquid medium

supplemented with chloramphenicol (12.5 µg/ml), kanamycin (50 µg/ml), 5NAA (100 µM), and 1x Fosmid CopyControl Induction Solution (Epicentre Biotechnologies, Madison, WI) at 30°C for 17 hours. After growth, microplates were centrifuged and supernatants were removed. BLK liquid medium (4) (200 µl) supplemented with 5NSA (200 µM) and 1x Fosmid CopyControl Induction Solution was added to each well, followed by incubation for 48 hours at 30˚C. Mutants unable to release nitrite from 5NSA were sequenced with Ez-Tn5 <KAN-2> specific outward reading primers (Ez-Tn5 <KAN-2> FP-1 forward primer and Ez-Tn5 <KAN-2> RP-1 reverse primer, Epicentre Biotechnologies, Madison, WI) by Nevada Genomics Center (Reno, NV).

4.3.3 Cloning of 5NAA deaminase (NaaA), 5NSA dioxygenase (NaaB), and lactonase (NaaC)

naaA, *naaB* or *naaC* were amplified by PCR using the appropriate primers (Table 4.1) (Integrated DNA Technologies, Coralville, IA) and GoTaq Flexi DNA Polymerase (Promega, Madison, WI) for *naaA* and *naaB* or DeepVent proofreading polymerase (New England Biolabs, Ipswich, MA) for *naaC*. The purified PCR products were ligated into BamHI and XhoI sites of the pET-21a vector (Invitrogen Corp., Carlsbad, CA). The resulting recombinant plasmids pJS803, pJS804, and pJS805 were transformed into *E.coli* DH5α (New England Biolabs, Ipswich, MA; Table 4.1) to maintain the plasmid or *E.coli* Rosetta 2(DE3)pLysS Competent Cells (Novagen; Table 4.1) for over-expression according to the manufacturers' protocols. The resulting inserts (Table 4.1) were sequenced with the T7 and T7 term primers by Genewiz Inc. (New Jersey) to verify the absence of mutations.

4.3.4 Over-expression of *naaA*, *naaB*, and *naaC*

Single colonies of *E. coli* Rosetta 2 pJS803, pJS804, or pJS805 were transferred into LB medium supplemented with ampicillin (100 μg/ml) and glucose (1%) and incubated for 17 hours at 37°C with shaking. Cells were subcultured into fresh medium (1:20, vol/vol) and incubated until the OD_{600} was 0.5-0.8. Expression of genes was induced by adding isopropyl β-D-1-thiogalactopyranoside (IPTG, 200 μg/ml) followed by incubation at 30˚C with shaking for 2-3 hours. The induced cells were harvested by centrifugation (10,000 × *g*) for 10 min at 4˚C, washed with ice-cold potassium phosphate buffer (pH 7.0, 20 mM), and stored at -80˚C until used.

4.3.5 Preparation of lactones (2-oxo-3-(5-oxofuran-2-ylidene)propanoic acid, or 4-carboxymethylenebut-2-en-4-olide)

To make lactones, induced cells of *E.coli* pJS804 were washed with phosphate buffer, suspended in the same phosphate buffer supplemented with 5NSA (500 μM), and incubated with shaking at 30°C until production was complete. Cells were removed by centrifugation (20,000 x g, 4°C, 10 min), and the supernatant was stored at -80˚C until used in enzyme assays. Approximately 85% of the lactones remained after 15 days of storage at -80˚C.

4.3.6 Enzyme assays

Cell pellets were suspended in ice-cold potassium phosphate buffer (20 mM, pH 7.0) and passed twice through a French pressure cell at 20,000 lb/in^2. Cell debris was removed by centrifugation (20,000 x g, 4°C, 20 min). Enzyme assays were carried out at 30°C in potassium phosphate (pH 7.0, 20 mM) or HEPES buffer (pH 7.4, 50 mM). Assay mixtures contained 0.01-1.5 mg/ml of protein and 5NAA (100-200 μM), 5NSA (400-500 μM) or lactones. External cofactors were not required. After appropriate intervals, trifluoroacetic acid (TFA) was added (1:100, vol/vol) to stop the reactions. The acidified reaction mixtures were clarified by centrifugation before HPLC analysis or colorimetric assays.

4.3.7 Transformation of 5NSA by intact cells

E.coli pJS800 was grown in LB medium supplemented with chloramphenicol (12.5 μg/ml), 1x CopyControl Fosmid Autoinduction Solution (Epicentre Biotechnologies, Madison, WI), and 5NAA (100 μM) at 30°C with shaking for 17 hours. Induced cells were harvested by centrifugation (10,000 × *g*, 10 min, 4˚C), washed twice with ice-cold potassium phosphate buffer (pH 7.0, 20 mM), and suspended in the same phosphate buffer. 5NSA was added to initiate the reaction, and the mixture was incubated at 30°C with shaking. Samples were taken at appropriate intervals, mixed with TFA (1:100, vol/vol) to stop the reactions, and clarified by centrifugation at 16,100 x g for 3 min prior to HPLC analysis.

4.3.8 Analytical methods

5-Chlorosalicylic acid (5CSA), and lactone (2-oxo-3-(5-oxofuran-2-ylidene)propanoic acid) were analyzed by high performance liquid chromatography (HPLC) using the method described previously for 5NAA and 5NSA (31). 5CSA and lactones were monitored at 305 nm (retention times [RT]: 3.5 min, lactone 1; 4.6 min lactone 2; 8.4 min, 5CSA). Fumarate was analyzed by HPLC with an ion exclusion column (ICSep ICE-ION-310 FAST Transgenomic, 6.5 mm by 150 mm) with an isocratic mobile phase of 1.74 mM H_2SO_4 at a flow rate of 0.3 ml/min over a period of 15 min, and detected at 210 nm (RT, 12.1 min).

Ammonia (30) and nitrite (39) were measured as reported previously. Chloride was analyzed according to a colorimetric method (2). Protein concentration was determined with a Pierce BCA Protein Assay Kit (Rockford, IL).

4.3.9 Chemicals

5-Chlorosalicylic acid (5CSA) was from TCI America (Portland, OR). Fumaric acid was from Chem Service (West Chester, PA). 5-Nitroanthranilic acid (5NAA) was from Sigma-Aldrich (Milwaukee, WI). 5-Nitrosalicylic acid (5NSA) was from Eastman Kodak (Rochester, NY).

4.3.10 Nucleotide sequence accession number

Nucleotide sequence of the DNA fragment containing the complete operon involved in the biodegradation of 5NAA was deposited in GenBank under the accession number GU188569.

4.4 RESULTS

4.4.1 *In silico* analysis of the genes involved in 5NAA degradation by *Bradyrhizobium* JS329

All of the genes involved in conversion of 5NAA to small organic acids are located on a 40 kb fosmid clone (pJS800, Table 4.1) from *Bradyrhizobium* sp. Strain JS329 (31). To identify the genes involved and their arrangement, pJS800 was sequenced. The assembled sequence comprised three gene clusters, one of which (Fig. 4.1) contained *naaA* and *naaB* identified previously as encoding 5NAA aminohydrolase (NaaA) and 5-nitrosalicylate dioxygenase (NaaB) (31). Based on BLASTP analysis, the deduced amino acid sequence of ORF2 encoding NaaA is distantly related to M20 peptidases (31), and contains metal-binding residues. ORF3 encoding NaaB is distantly related to salicylate dioxygenase, gentisate dioxygenase, and 1-hydroxy-2-naphthoic acid dioxygenase (31). It contains conserved histidine residues essential for binding the ferrous ion in the catalytic site (13, 31, 48). The deduced amino acid sequence of ORF5 contains a conserved domain in common with dienelactone hydrolase and related enzymes (COG0412) (24) but the overall sequence has less than 33% identity to that of the nearest relative (Fig. 4.1). The deduced amino acid sequence of ORF6 has 68% amino acid identity to "fumarylacetoacetate (FAA) hydrolase" from *Paracoccus denitrificans* PD1222 (NCBI accession number: YP_913985.1) based on BLASTX results. The enzyme contains conserved domains similar to those of the FAA hydrolase (pfam01557) and isomerase (TIGR02303, TIGR02305) involved in keto-enol isomerization of 2-oxohept-3-ene-1,7-

dioate (24). The findings are consistent with fumarate and lactone as intermediates in the degradation pathway of 5NAA.

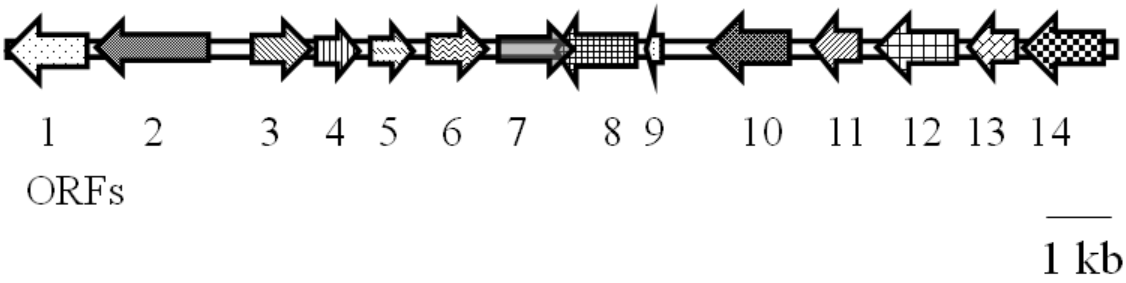


Fig. 4.1 Map of the gene cluster containing the operon involved in the degradation of 5NAA by *Bradyrhizobium* JS329. The amino acid identity to the best match from BLASTP search and the corresponding NCBI accession number are shown in parentheses as follows: 1,4-hydroxybenzoate transporter (46%, ZP_00946270.1); 2, 5NAA deaminase (100%, GU188569.1); 3, 5NSA dioxygenase (100%, GU188569.1); 4, Rieske 2Fe-2S family protein (35%, ZP_01125630.1); 5, carboxymethylenebutenolidase (33%, NP_926347.1); 6, fumarylacetoacetate (FAA) hydrolase (68%, YP_913985.1); 7, unknown function; 8, succinate dehydrogenase (77%, ZP_01998497.1); 9, short-chain dehydrogenase/reductase (72%, YP_002496152.1); 10, 2-oxo-hept-3-ene-1,7-dioate hydratase (55%, ZP_05966920.1); 11, translation initiation inhibitor (47%, ZP_00946594.1); 12, 3-hydroxyanthranilate 3,4-dioxygenase (49%, YP_509389.1); 13, unknown function; 14, ferredoxin reductase (40%, ZP_01224350.1).

4.4.2 Properties of NaaA

5NAA deaminase (NaaA) is responsible for an unusual hydrolytic deamination of 5NAA to produce 5NSA in *Bradyrhizobium* JS329 (31). To test the substrate specificity and provide insight about why aminohydrolase and not dioxygenase enzymes catalyze the initial attack, we determined the activity toward several structural analogs of 5NAA (Fig. 4.2). No activity was detected with anthranilic acid, 4-nitroaniline, aniline, 5-hydroxyanthranilic acid and 4-nitroanthranilic acid (Fig. 4.2), which indicates that both nitro and carboxyl groups on the aromatic ring are essential for 5NAA deamination.

The optimum pH and temperature were 7.2 and 42-45°C. Addition of the metal ion chelators EDTA (1 mM), 2,2'-dipyridyl (1 mM), and *o*-phenanthroline (1 mM) inhibited the activity of NaaA by 99%, 70%, and 98%. The activity was restored by addition of divalent metals Co^{2+} (100%), Mn^{2+} (85%), Zn^{2+} (81%), Fe^{2+} (67%) or Ni^{2+} (39%). In crude extracts Mn^{2+} stimulated enzyme activity the most (data not shown), which suggested that Mn^{2+} plays a physiological role. The metal requirement and metal-binding residue(s) will be determined rigorously when NaaA is purified for structural analysis. Ultrafiltration of the overexpressed NaaA did not inhibit the enzyme activity, consistent with our earlier observations with crude extracts from *Bradyrhizobium* JS329 (31), which indicated that the divalent metal was bound to the enzyme. NaCl (5 mM) inhibited the activity of NaaA by 39%, suggesting that ionic strength affects the interaction between the divalent metal with the amino acid ligand(s) or conformation of the enzyme.

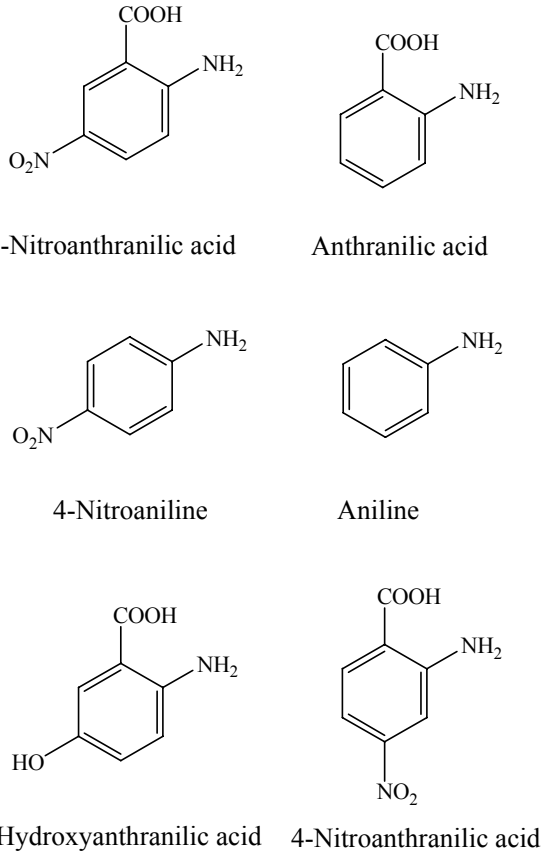


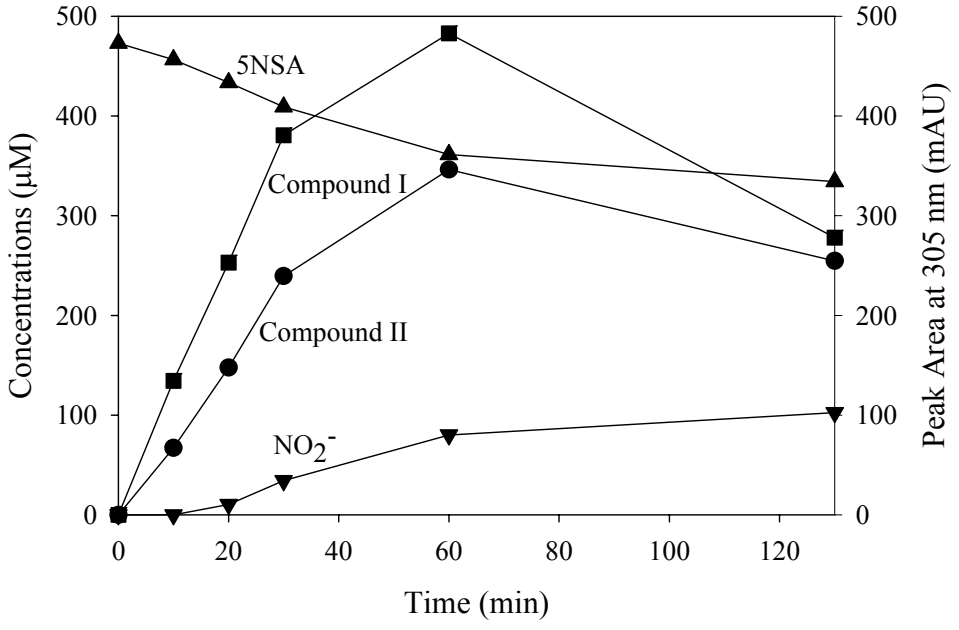
Fig. 4.2 5NAA and its analogs.

4.4.3 Mutagenesis of pJS800

Nitrite was released during the oxidation of 5NSA by *E.coli* pJS800 and when *Bradyrhizobium* JS329 grew on 5NAA or 5NSA (31). To identify the gene involved in removal of the nitro group as nitrite, fosmid pJS800 was subjected to Tn*5* transposon mutagenesis. Mutants that lost the ability to release nitrite during the transformation of 5NSA were sequenced. Tn*5* transposons were inserted only into *naaB*, which is responsible for the ring cleavage of 5NSA (31). The results indicate that the release of nitrite takes place during the ring fission reaction or spontaneously after ring-fission.

4.4.4 Transformation of 5NSA by enzymes in cell extracts

In *Bradyrhizobium* JS329, 5NSA is oxidized by 5-nitrosalicylate dioxygenase (NaaB) encoded by ORF3 (Fig. 4.1) (31). 5-Nitrosalicylate dioxygenase is distantly related to gentisate dioxygenase from *Oligotropha carboxidovorans* OM5 (31). In *Pseudaminobacter salicylatoxidans* gentisate dioxygenase catalyzes the conversion of 5-chlorosalicylate to a ring-rission product that undergoes spontaneous lactonization accompanied by elimination of Cl^- (13). We tested whether NaaB catalyzed similar reactions with 5-nitro- and 5-chlorosalicylate. Transformation of 5NSA or 5CSA in cell extracts from *E.coli* containing pJS804 was accompanied by accumulation of nitrite or chloride and two unknown products (Fig. 4.3) with identical UV spectra (A_{max}=300 nm). The formation, spectra and behavior of the two unknown compounds were identical to those of the lactones previously reported to be produced from 5-chlorosalicylate by gentisate dioxygenase (13). The products were not further transformed, but slowly decomposed.



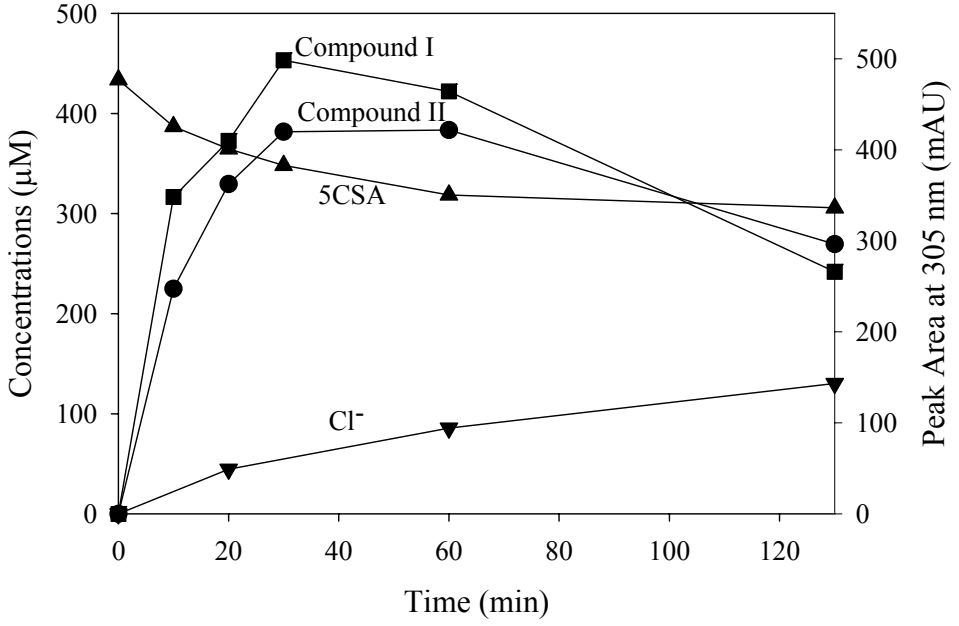


Fig. 4.3 Biotransformation of 5NSA (upper) and 5CSA (lower) by cell extracts from *E.coli* pJS804. Reaction mixtures contained 1.3 mg/ml of protein.

pJS805 was constructed to express ORF5, the putative lactone hydrolase. When extracts of cells containing pJS805 were incubated with the unknown compounds produced from 5NSA or 5CSA both compounds disappeared rapidly (Fig. 4.4), accompanied by an increase of absorbance at 332 nm (data not shown) characteristic of formation of maleylpyruvate (A_{max}=330-334 nm) (10, 48). The results are consistent with a dioxygenase-catalyzed ring-opening, followed by spontaneous lactonization and nitrite elimination, and enzyme-catalyzed hydrolysis of the lactone to produce maleylpyruvate (13).

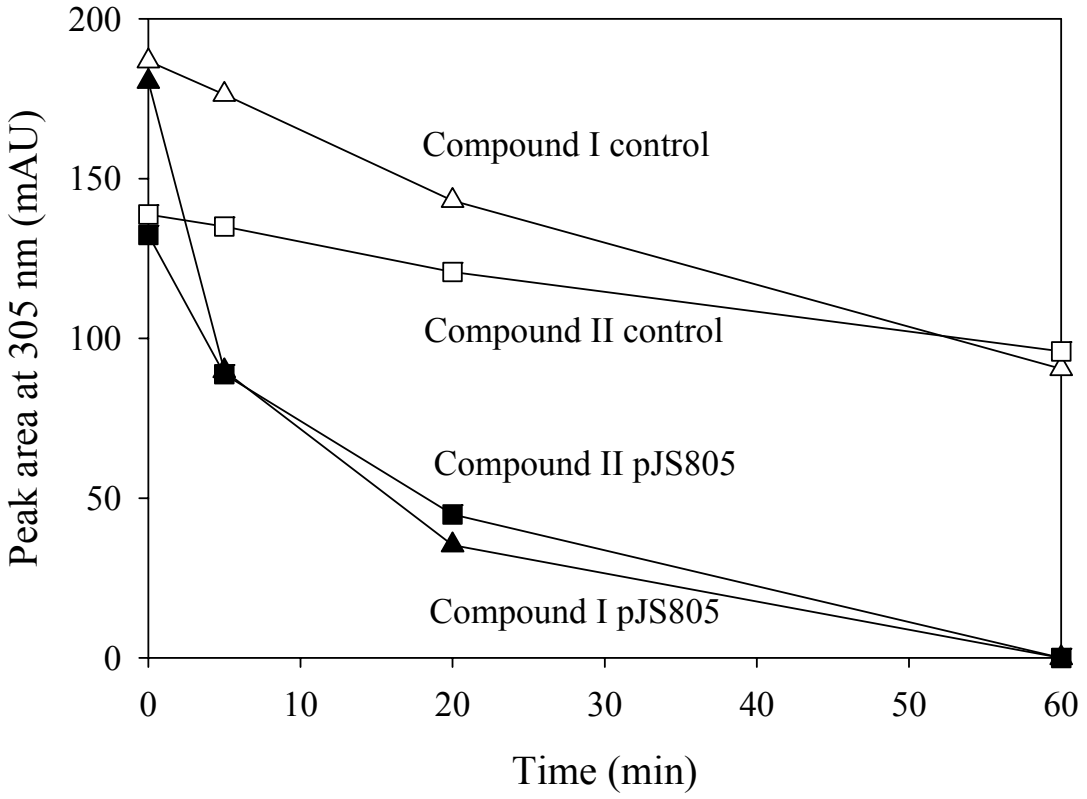


Fig. 4.4 Biotransformation of lactones by cell extracts from *E.coli* pJS805. Reaction mixtures contained 0.015 mg/ml of protein. ■, peak area of compound II for *E.coli* pJS805; ▲, peak area of compound I for *E.coli* pJS805; □, peak area of compound II for the abiotic control; Δ, peak area of compound I for the abiotic control.

4.4.5 Subsequent steps in the pathway

Maleylpyruvate is the key intermediate in the biodegradation of 5-substitituted salicylates including 5-hydroxysalicylate (gentisate) (48) and 5-halosalicylates (13). The conversion of maleylpyruvate to central intermediates is well established for a variety of bacteria (48), and involves either a) isomerization to fumarylpyruvate followed by hydrolysis to fumarate and pyruvate, or b) direct hydrolysis to maleate and pyruvate (16). To determine which reaction is involved in the lower pathway of 5NSA degradation,

E.coli pJS800 was incubated with 5NSA. Transient accumulation of fumarate during the transformation (data not shown) and the presence of the gene encoding fumarylacetoacetate hydrolase (ORF6, Fig. 4.1) suggest that maleylpyruvate is transformed in *Bradyrhizobium* JS329 via fumarylpyruvate. Although the gene encoding maleylpyruvate isomerase is absent in the fosmid pJS800, it is a key gene involved in the metabolism of tyrosine and present in all sequenced genomes of *Bradyrhizobium* (NCBI GeneID: 1054074, 5151487, or 5115165) or *E. coli* (NCBI GeneID: 6968260). The details and the potential for enzymes of the alternative pathway to be encoded elsewhere in the genome were not investigated.

4.5 DISCUSSION

The biodegradation mechanism of 5NAA is a departure from the typical dioxygenase-catalyzed removal of amino (43), nitro (29), and carboxyl (11) substituents from the benzene ring. Amino functional groups on aliphatic and heterocyclic compounds can be removed by hydrolases (23, 36); however, to our knowledge, 5NAA deaminase is the first hydrolase that can catalyze the hydrolytic removal of an amino group attached to the benzene ring. It seems likely that the $-NO_2$ and -COOH groups synergetically polarize 5NAA due to the electronic effect, which makes the amino group of 5NAA equivalent to an "imino group", and thus facilitates the hydrolysis. The effects might similarly inhibit dioxygenase attack. Insight about whether the mechanism is widespread will require evaluation of pathways for biodegradation of other substituted anilines including nitroanilines and chloroanilines. In several isolates able to grow on 4-chloroaniline the degradation pathway seems to be initiated by aniline dioxygenase to produce

chlorocatechol, followed by dioxygenase-catalyzed *ortho*-cleavage of the benzene ring (45, 47). Therefore, the hydrolytic deamination mechanism seems not to apply to the catabolism of chloroanilines.

Although they show no significant identity to any biochemically characterized enzymes, the properties of 5NAA deaminase resemble those of the M20 family peptidases (14, 34, 37) or acetylornithine deacetylase (17) in the requirement for transition metal ions, conserved metal-binding residues, inhibition by EDTA and 1,10-phenanthroline, and activity at relatively high temperature. The M20 family comprises thousands of sequences of dipeptidases, carboxypeptidases, and aminopeptidases from archaea, bacteria and eukaryotes (http://merops.sanger.ac.uk/). By analogy to the well characterized dipeptidase (19) and acetylornithine deacetylase (38) mechanisms, our hypothesis for the mechanism of 5NAA deamination is that the divalent metal ion serves to stabilize the conformation of the enzyme and activate water for the hydrolytic reaction. Determination of the structure and mechanism of 5NAA deaminase is currently in progress.

Biodegradation of 5NSA is one of several examples where a nitrophenol serves as the ring-fission substrate without prior removal of the nitro group (9, 27, 31). Determination of the downstream pathway not only reveals the complete degradation pathway of the natural nitroaniline (5NAA), but also sheds light on the potential mechanism involved in degradation of other important synthetic nitro compounds such as picric acid and 2,6-DNT. The fact that Tn*5* insertion into *naaB* disrupted nitrite release indicates that the ring-fission dioxygenase is involved in the denitration, but does not establish the mechanism. The mechanism of gentisate dioxygenase catalyzed elimination

of Cl^- from 5-chlorosalicylate is well established, however (13). Gentisate 1,2-dioxygenase from *Pseudaminobacter salicylatoxidans* catalyzes the oxidative ring-cleavage of 5-halosalicylates and produced a lactone with the simultaneous release of HX, followed by abiotic hydrolysis of the lactone to form maleylpyruvate (12-13).

Although gentisate dioxygenase did not attack 5NSA (13) the 5NSA dioxygenase clearly catalyzes an analogous reaction with both 5NSA and 5-chlorosalicylate. The products are identical and identical to the lactones produced from 5-chlorosalicylate by gentisate dioxygenase (13). When the lactone carries an alkene side chain it exists as *cis* and *trans* isomers with identical spectra but different retention times in HPLC (35). Therefore, according to our results and by analogy to previous studies, the initial ring-fission product of 5NSA dioxygenation (not detected by HPLC) was spontaneously and rapidly cyclized to form lactones accompanied by the release of NO_2^- (Fig. 4.5).

A substantial amount of previous work (20) suggests that the nitro group is removed from nitro compounds as nitrite by monooxygenases (21, 29), dioxygenases (29, 41) or reductases that attack the aromatic ring (9, 29, 32). To our knowledge, this is the first report that the nitro group can be spontaneously released as nitrite during formation of lactones from ring fission products.

It now seems that salicylate (13, 31) and a wide variety of 5-substituted salicylates (8, 13, 42) including 5-amino-, 5-halo-, 5-methyl-, 5-nitro-, 5-hydroxysalicylate can be transformed by enzymes related to salicylate/gentisate 1,2-dioxygenases to open the aromatic rings. When the 5 position is substituted with a halogen or nitro group, the spontaneous lactone formation is accompanied by the elimination of HX or HNO_2. A recently described exception involves biodegradation of 5-chlorosalicylate initiated by

salicylate 1-hydroxylase catalyzed oxidation to produce 5-chlorocatechol (26). There is considerable precedent during the biodegradation of chlorocatechols including 3-chlorocatechol, 4-chlorocatechol, 5-chlorocatechol, 3,5-dichlorocatechol, and 3-methyl-5-chlorocatechol (7, 25-26, 33) for the formation of lactones accompanied by the release of halide and subsequent enzymatic hydrolysis of the ring to form intermediates of central metabolism. It remains to be seen whether the nitro group of nitrocatechols can be eliminated by an analogous lactonization of the ring fission product.

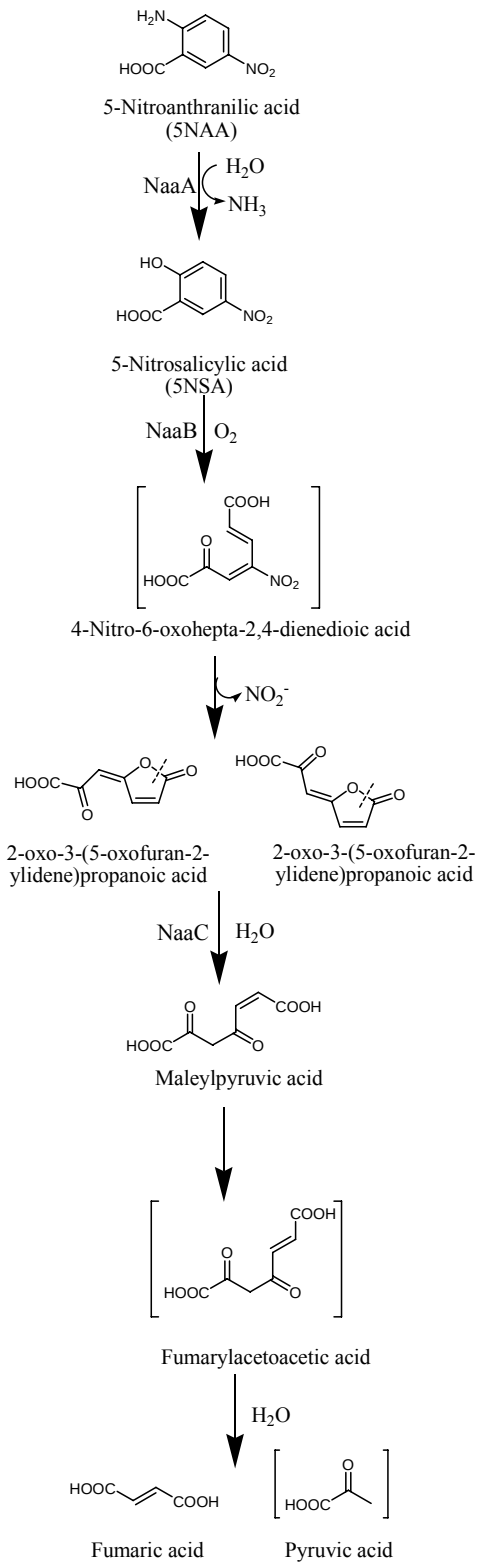


Fig. 4.5 Proposed degradation pathway for 5NAA in *Bradyrhizobium* JS329.

Based on the sequence analysis, biochemistry studies, and analogy with the mechanism of 5-chlorosalicylate degradation by gentisate dioxygenase (13), we propose the biodegradation pathway for 5NAA in *Bradyrhizobium* JS329 (Fig. 4.5). The initial reaction is a hydrolytic removal of the amino group to form 5NSA which is then oxidized by 5NSA dioxygenase to open the aromatic ring. The resulting ring-fission product undergoes spontaneous lactonization accompanied by the removal of the nitro group as nitrite. The lactone ring opening is catalyzed by lactone hydrolase to form maleylpyruvate, which is in turn hydrolyzed to produce intermediates of central metabolism.

Biodegradation pathways of synthetic compounds seem to have evolved from those of natural analogs (6, 18, 44, 46). Only a few biodegradation pathways have been established for natural nitro compounds (20), so our current understanding stops far short of enabling predictions about evolution of specific pathways. Recent work including this study suggests that detailed characterization of the metabolic pathways and enzymes can uncover new metabolic diversity (31), reveal the genes that enabled the rapid and recent evolution of pathways for degradation of synthetic nitro compounds, and provide insight about the most fruitful area of inquiry to attack the problem of the many unknown ORFs (31) and incorrectly annotated genes (28) .

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CHAPTER 5

Conclusions and Perspectives

Biodegradation of synthetic compounds has been studied extensively, but little is known about the metabolic diversity required for catabolism of naturally occurring nitro compounds. Evolution of strategies for biodegradation of synthetic organic pollutants relies on recruitment of genes from catabolic pathways for natural compounds. The goal of this work was to discover novel metabolic diversity involved in biodegradation of natural nitro compounds. In the thesis work two new enzymes, 2NI nitrohydrolase and 5NAA aminohydrolase, were discovered to be involved in the initiation of biodegradation pathways for 2NI and 5NAA, respectively. The findings support our hypothesis that the study of the metabolism of natural organic compounds, selected on the basis of unusual structural features and/or ecological roles, can reveal new metabolic diversity. The gene encoding 2NI nitrohydrolase conferred drug resistance to 2NI when heterologously expressed in *E. coli*. The finding suggests that the discovery of novel metabolic diversity involved in biodegradation of natural antibiotics can identify the origin of novel drug resistance mechanisms.

5.1 SUMMARY AND FUTURE WORK ON THE BIODEGRADATION OF 2NI

2NI, produced by pseudomonads and *Actinomycetes*, along with a wide variety of analogs (See Chapter 1), are clinically important prodrugs. Enzymatic reduction of the nitro group to form the corresponding radical, nitroso or hydroxylamine intermediates is

required for activation of the prodrug. The initial step in the biodegradation of 2NI by a soil bacterium, *Mycobacterium* JS330, is a hydrolytic denitration to produce imidazole-2-one and nitrite, which prevents the formation of toxic intermediates and thus eliminates the antibiotic effect of 2NI. Sequence analysis reveals that the gene encoding 2NI nitrohydrolase is deeply branched. To our knowledge no nitrohydrolase has been reported previously. The results, therefore, not only revealed a novel nitrohydrolase, but also provide the first clear evidence for the initial biodegradation pathway of nitroheterocycles.

Facile detection of the ability to degrade 2NI in natural ecosystems suggests an important ecological role for 2NI. It is not clear whether 2NI acts as a weapon or a signaling molecule for bacteria, but previous studies provided convincing evidence that many antibiotics are produced mainly for bacterial communication (2).

The wide distribution of 2NI degrading bacteria in soil raises questions about the diversity of genes encoding 2NI nitrohydrolases and the mechanism of 2NI denitration. In the future the genes encoding 2NI nitrohydrolase will be identified to enable the rational design of molecular probes, which will then be used to determine the distribution of the drug-resistance genes in natural ecosystems. In addition, to understand how 2NI nitrohydrolase works, it will be necessary to know not only the protein sequence, but also the structure of the active site. X-ray crystallography can provide much more information on the 3D structure of proteins than any other technique. Sufficient amounts of purified enzyme will be crystallized to determine its 3D structure by X-ray analysis to reveal the details of the mechanism at the active site. The study on docking interactions between substrates and pocket of the enzyme will provide the basis for predicting the

biodegradation of nitro heterocyclic pollutants and enable development of strategies for overcoming resistance to 2NI and its analogs.

Discovery of a new hydrolytic denitration mechanism raises the question whether this type of reaction is involved in denitration of all or most synthetic nitroheterocycles. Therefore, some model synthetic compounds, such as the explosive 2,4-dinitroimidazole, will be chosen to study their biodegradation pathways. Detailed characterization of the reaction kinetics and mechanism can provide insight into strategies for decontamination and/or storage of explosives.

5.2 SUMMARY AND FUTURE WORK ON THE BIODEGRADATION OF 5NAA

5NAA is produced by *Streptomyces scabies* (the causative agent of potato scab) by an unknown mechanism (7), and degraded by a *Bradyrhizobium* sp. isolated from potato-farm soil. Such facts suggest a spatial link between the production and degradation of 5NAA, and an important but currently unknown ecological role of 5NAA in potatoes.

We determined the biochemical and genetic mechanisms for the biodegradation of 5-nitroanthranilic acid (5NAA) in *Bradyrhizobium* JS329. The biodegradation pathway of 5NAA is initiated by an unusual hydrolytic deamination. The corresponding gene is very distantly related to biochemically characterized genes in the NCBI database. The nitro group of 5NAA is eliminated as nitrite during the spontaneous formation of lactones from a ring fission product, analogous to removal of the chloro group from 5-chlorosalicylate by *Pseudaminobacter salicylatoxidans*.

The discovery of the novel hydrolytic deaminase in the degradation pathway of 5NAA raises questions about the mechanism for 5NAA deamination. Since the gene

encoding 5NAA deaminase is deeply branched, bioinformatic analysis alone is not sufficient to elucidate the mechanism. Therefore, ongoing experiments will elucidate the structure of 5NAA deaminase by X-ray crystallography to reveal the active sites.

In addition, the initial steps in the biodegradation of 4-nitroaniline, a synthetic analog of 5NAA, should be determined. Bacteria able to grow on 4-nitroaniline were isolated and the pathway is under investigation. Elucidation of the mechanism of biodegradation of 4-nitroaniline may reveal the evolutionary relationships among degradation pathways for nitroanilines.

5.3 SIGNIFICANCE

Environmental microorganisms rapidly evolved the ability to readily degrade synthetic pollutants released into the biosphere in the past century. The process was accomplished by recruitment of gene(s) involved in biodegradation pathways for natural organic compounds by horizontal gene transfer (3, 12). Several examples are known (6), but our current understanding of metabolic diversity involved in metabolism of myriad natural organic compounds fails to accurately predict specific pathways for synthetic pollutants. The thesis work to determine biodegradation pathways for model natural nitro compounds 5NAA and 2NI has revealed a surprising diversity of new enzyme catalyzed reactions. The genes might have provided the basis of recent evolution of bacteria that degrade synthetic pollutants containing nitro group(s), particularly amino nitroaromatic and nitroheterocyclic explosives.

Prior to the thesis work four strategies (monooxygenation, dioxygenation, nitroreduction, and hydride-Meisenheimer formation) were known for initial attack on

nitro aromatic compounds by bacteria, one strategy (monooxygenation catalyzed by P450) was known for denitration from N-NO_2 compounds, and two strategies (monooxygenation and oxidation) were known for nitro aliphatic compounds (See Chapter 1 for details). The work described here revealed two additional strategies, hydrolytic denitration and nitrite release during lactone formation, which thus enriched our biochemical understanding of denitration mechanisms.

The current thesis work also revealed a new deamination mechanism, hydrolytic amino group removal from a phenyl ring. The introduction of amino group(s) into nitro aromatic compounds can make explosives more thermally stable. For example, the thermodynamic stability increases in the order of mono-amino-2,4,6-trinitrobenzene (MATB), 1,3-diamino-2,4,6-trinitrobenzene (DATB), and 1,3,5-triamino-2,4,6-trinitrobenzene (TATB) (1). The understanding of hydrolytic deamination from this thesis work might be applicable to deamination mechanism of such amino nitroaromatic explosives.

Substantial amounts of previous work have been conducted to understand the biodegradation mechanisms of nitro aromatic compounds, but nothing was known about such mechanisms for nitroanilines and nitroheterocycles. This work provides the first example for biodegradation of both groups of molecules on the basis of biochemistry and molecular biology. Degradation pathways of 5NAA and 2NI serve as precedents for those of nitroaniline and nitroheterocyclic pollutants.

Nothing was known about the mechanism of nitro group removal from an aliphatic ring fission product of a nitro aromatic compound. For example, the degradation pathways of both 2,6-DNT (9) and picric acid (4) result in the formation of two nitro

aliphatic intermediates, HNPA (2-hydroxy-5-nitropenta-2,4-dienoic acid) and 4,6-DNH (4,6-dinitrohexanoate), with unknown mechanisms of subsequent denitration (See Chapter 4). Elucidation of the mechanism for removal of the nitro group during the biodegradation of 5NAA not only enriches our understanding of catabolism of nitro aliphatic compounds, but also serves as a precedent for prediction of catabolic pathways of related compounds such as 2,6-DNT or picric acid.

The genes encoding 5NAA aminohydrolase and 2NI nitrohydrolase were not significantly related to any previously biochemically characterized genes, which means that both are new functional annotations. Bioinformatics alone is ineffective to discover new enzymes, because it provides annotations based on previously characterized genes and often provides misleading information perpetuated from initial errors (10). The work described here provides an example that it is comparatively easier to find new genes by discovering the activities first instead of the sequences first.

The discovery of the 2NI nitrohydrolase also provides clear evidence for a new mechanism of drug resistance. To our knowledge this is the first experimental evidence of a new mechanism of drug resistance in soil prior to its appearance in pathogens. This work indicates that studies of the biodegradation of natural antibiotics can not only reveal novel metabolic diversity, but also deepen our understanding of drug resistance in the environmental reservoir.

5.4 REMAINING QUESTIONS

Although the thesis work solved some mysteries, it raised new questions that are important for environmental microbiologists, biochemists, and/or bioinformaticians. To

tackle the problems, collaboration among investigators with diverse backgrounds might be necessary.

1) What are the evolutionary relationships of aminohydrolases or nitrohydrolases involved in biodegradation of synthetic chemical pollutants?

The work reveals two novel enzymes “5NAA aminohydrolase” and “2NI nitrohydrolase”. 5NAA and 2NI represent a variety of synthetic amino nitroaromatic compounds and nitroheterocycles, respectively, and these two groups of synthetic chemicals have wide applications (See Chapter 1). Because both 5NAA aminohydrolase and 2NI nitrohydrolase have very narrow substrate ranges, discovery and characterization of other enzymes homologous to 5NAA aminohydrolase or 2NI nitrohydrolase involved in the biodegradation of synthetic chemicals are required to reveal phylogenetic relationships and conserved amino acid residues of both types of enzymes. The molecular information, particularly conserved regions of enzyme sequences will enable the design of PCR primers to detect the presence of genes with the potential to degrade synthetic pollutants.

2) What is the most efficient way to discover novel metabolic diversity?

Due to the absence of high-throughput strategies an *in silico* metabolic prediction approach (12) based on computational metabolic rules provides a compelling solution to elucidating the biodegradation mechanisms of myriad organic compounds. However, novel metabolic diversity must be included in the predictive systems to ensure the correct metabolic predictions. The high throughput “omics” technologies alone are often

ineffective at discovery of new enzymes due to their reliance on what is known. Relatively simple and straightforward experiments, as we have done for 5NAA aminohydrolase and 2NI nitrohydrolase, can reveal novel genes and novel biochemical reactions. Identification of novel genes merely based on functional assays is time-consuming (It takes conservatively one year for one investigator to determine the degradation pathway for one compound), however, and chemicals must be available to test activities of degrading bacteria and/or genes. Therefore, development of a novel high throughput approach is needed to discover the novel metabolic diversity involved in metabolism of the myriad natural compounds.

3) What is the most effective way to functionally annotate the unknown ORFs in genetic databases?

Enormous numbers of unknown and misidentified ORFs exist in the bacterial genome database. Even for the well-studied *E. coli*, around 40% of the ORFs have unknown functions (5, 8). Recently, functional tests of mutagenized unknown/putative ORFs by Phenotype MicroArray technology (http://www.biolog.com/), sequence/structure-based strategy (http://enzymefunction.org/), and comparative genomic analysis have been employed to tackle this problem, but each one has its limitations. In addition, investigations are mainly focused on functional annotation of the unknown/putative genes from model strains, particularly *E. coli*, but a number of genes present in environmental microorganisms are likely to be more important. Therefore, continuing efforts are required to deal with this huge challenge for modern biology.

4) Why are some of the resistance markers in the environmental reservoir not present in pathogens?

The gene encoding 2NI nitrohydrolase is widespread in the environment, but has not been reported in pathogens. The observation is consistent with the findings that drug resistance genes revealed by metagenomic sequencing of the microbiome from healthy humans are distantly related to those from pathogens, which might reflect an unknown barrier to lateral gene transfer *in vivo* (11). Deeper investigation of the mechanism of gene acquisition could help control and predict the dissemination of drug resistance markers.

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