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Shirley F Nishino, Jim C Spain, Sarah H Craven, Johana Husserl, Zohre Kurt, Samantha T Parks, Rayford B Payne, Yi Qu, Kwanghee A Shin

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14. ABSTRACT

The biosynthesis and biodegradation of natural products may be the source of many of the unknown and hypothetical genes being revealed by genomics and metagenomics. The biodegradation pathways of a range of naturally occurring compounds structurally related to compounds of interest to DoD were elucidated along with the genes and enzymes responsible for the catabolic reactions. The purpose was to explore the metabolic diversity of catabolic pathways for natural compounds as the potential source of genetic content that has led to the recent, rapid

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Metabolic Diversity for Degradation, Detection, and Synthesis of Nitro Compounds and Toxins

ABSTRACT

The biosynthesis and biodegradation of natural products may be the source of many of the unknown and hypothetical genes being revealed by genomics and metagenomics. The biodegradation pathways of a range of naturally occurring compounds structurally related to compounds of interest to DoD were elucidated along with the genes and enzymes responsible for the catabolic reactions. The purpose was to explore the metabolic diversity of catabolic pathways for natural compounds as the potential source of genetic content that has led to the recent, rapid evolution of pathways for degradation of synthetic chemicals. Bacteria were isolated that are able to grow on the natural nitro compounds 3-nitrotyrosine, 3-nitropropionic acid, 1-nitro-2-phenylethane, diphenylamine, 5-nitroanthranilic acid, 2-nitroimidazole, chloramphenicol, N-nitroethylenediamine, 8-nitroguanine, and a natural organophosphate, (S)-cinnamoylphosphoramide. Other isolates were able to grow on the synthetic compounds nitroglycerin, 2,4-dinitroanisole, and 4-nitroaniline. In some cases new enzymes and enzyme functions were discovered, in one system the long-standing mystery of the physiological substrate of the enzyme encoded by a widespread gene was settled. Although only a limited sample of natural compounds was studied, the results indicated that catabolic pathways for natural oganic compounds comprise a previously untapped source of metabolic diversity with the potential for wide applications in biocatalysis for materials synthesis and destruction.

Enter List of papers submitted or published that acknowledge ARO support from the start of the project to the date of this printing. List the papers, including journal references, in the following categories:

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Received		Paper
2012/07/02 19	10	Kevin Francis, Shirley F. Nishino, Jim C. Spain, Giovanni Gadda. A novel activity for fungal nitronate monooxygenase: Detoxification of the metabolic inhibitor propionate-3-nitronate, Archives of Biochemistry and Biophysics, (05 2012): 84. doi: 10.1016/j.abb.2012.03.015
2012/07/02 19	7	Ronald Parry, Shirley Nishino, Jim Spain. Naturally-occurring nitro compounds, Natural Product Reports, (01 2011): 152. doi: 10.1039/c0np00024h
2012/07/02 18	11	J. Husserl, J. B. Hughes, J. C. Spain. Key Enzymes Enabling the Growth of Arthrobacter sp. Strain JBH1 with Nitroglycerin as the Sole Source of Carbon and Nitrogen, Applied and Environmental Microbiology, (03 2012): 3649. doi: 10.1128/AEM.00006-12
2012/07/02 18	6	J. Husserl, J. C. Spain, J. B. Hughes. Growth of Arthrobacter sp. Strain JBH1 on Nitroglycerin as the Sole Source of Carbon and Nitrogen, Applied and Environmental Microbiology, (01 2010): 1689. doi: 10.1128/AEM.02400-09
2012/07/02 18	9	Y. Qu, J. C. Spain. Molecular and Biochemical Characterization of the 5-Nitroanthranilic Acid Degradation Pathway in Bradyrhizobium sp. Strain JS329, Journal of Bacteriology, (04 2011): 3057. doi: 10.1128/JB.01188-10
2012/07/02 18	8	Yi Qu, Jim C. Spain. Catabolic pathway for 2-nitroimidazole involves a novel nitrohydrolase that also confers drug resistance, Environmental Microbiology, (04 2011): 1010. doi: 10.1111/j.1462-2920.2010.02406.x
2012/07/02 18	5	Y. Qu, J. C. Spain. Biodegradation of 5-Nitroanthranilic Acid by Bradyrhizobium sp. Strain JS329, Applied and Environmental Microbiology, (01 2010): 1417. doi: 10.1128/AEM.02816-09
2012/07/02 18	4	S. F. Nishino, K. A. Shin, R. B. Payne, J. C. Spain. Growth of Bacteria on 3-Nitropropionic Acid as a Sole Source of Carbon, Nitrogen, and Energy, Applied and Environmental Microbiology, (04 2010): 3590. doi: 10.1128/AEM.00267-10
2012/07/02 18	3	K. A. Shin, J. C. Spain. Pathway and Evolutionary Implications of Diphenylamine Biodegradation by Burkholderia sp. Strain JS667, Applied and Environmental Microbiology, (02 2009): 2694. doi: 10.1128/AEM.02198-08
2012/07/02 18	2	L. D. Rankin, D. M. Bodenmiller, J. D. Partridge, S. F. Nishino, J. C. Spain, S. Spiro. Escherichia coli NsrR Regulates a Pathway for the Oxidation of 3-Nitrotyramine to 4-Hydroxy-3-Nitrophenylacetate, Journal of Bacteriology, (07 2008): 6170. doi: 10.1128/JB.00508-08
2012/07/02 18	1	Thomas B. Hofstetter, Jim C. Spain, Shirley F. Nishino, Jakov Bolotin, Rene? P. Schwarzenbach. Identifying Competing Aerobic Nitrobenzene Biodegradation Pathways by Compound-Specific Isotope Analysis, Environmental Science & Technology, (07 2008): 4764. doi: 10.1021/es8001053

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1. Shin, K., and J. Spain. 2007. Biodegradation of diphenylamine, abstr. Q-014, Annual Meeting American Society for Microbiology, Toronto, Canada.
2. Payne, R., Y. Qu, S. Nishino, and J. Spain. 2007. Cloning and molecular characterization of the key denitrase involved in the catabolism of 3-nitrotyrosine, abstr. Q-013, Annual Meeting American Society for Microbiology, Toronto, Canada.
3. Nishino, S., R. Payne, and J. Spain. 2008. Pathway for bacterial degradation of 3-nitropropionic acid and the purification and characterization of a novel denitrase, abstr. Q-504, Annual Meeting American Society for Microbiology, Boston, MA.
4. Nishino, S. F., and J. C. Spain. 2008. Metabolic diversity for detection and destruction of natural analogs of explosives and chemical agents, abstr. T022, Chemical and Biological Defense Physical Science and Technology Conference, New Orleans, LA.
5. Nishino, S. F., Y. Qu, K. Shin, and J. C. Spain. 2009. Detection and destruction of natural analogs of explosives and chemical agents. abstr. Chemical and Biological Defense Science and Technology Conference, Dallas, TX.
6. Parks, S. T., Y. Qu, and J. C. Spain. 2009. Identification and characterization of DenA, a novel HNPA denitrase integral to 3-nitrotyrosine degradation in Variovorax sp. JS669, abstr. 95th Annual Meeting ASM Southeastern Branch Conference.
7. Qu, Y., and J. C. Spain. 2009. Biodegradation of 5-nitroanthranilic acid by a Bradyrhizobium sp., abstr. Q-268, Annual Meeting American Society for Microbiology, Philadelphia, PA.
8. Shin, K., and J. Spain. 2009. Biodegradation of diphenylamine by Burkholderia sp. strain JS667: pathway and evolutionary implications, abstr. Q-279, Annual Meeting American Society for Microbiology, Philadelphia, PA.
9. Spain, Jim C. 2009. Proctor & Gamble Award Lecture. Biodegradation of natural chemicals: assigning function to unknown orfs
10. Parks, S. T., S. Lee, and J. C. Spain. 2009. Biodegradation of 2-nitropropylbenzene, a synthetic analog of 1-nitro-2-phenylethane by Burkholderia sp. JS670. 95th Annual ASM Southeastern Branch Conference.
11. Parks, S. T., Y. Qu, and J. C. Spain. 2010. Identification of 4-hydroxy-3-nitrophenylacetate denitrase in Variovorax sp. JS669, abstr. First Southeast Enzyme Conference, Atlanta, GA.
12. Francis, K., S. Nishino, J. Spain, and G. Gadda. 2011. A novel activity for fungal nitronate monooxygenase: detoxification of the metabolic inhibitor propionate-3-nitronate, abstr. Presented at the Second Southeast Enzyme Conference, Atlanta, GA.
13. Kurt, Z., Y. Qu, E. Lang, and J. C. Spain. 2012. Biodegradation of 4-nitroaniline by a Rhodococcus sp. abstr. Q-425, Annual Meeting American Society for Microbiology, San Francisco, CA.
14. Nishino, S. F., and J. C. Spain. 2012. Growth of marine bacteria on the natural organophosphate (S)-cinnamoylphosphoramide. abstr. Q-1497, Annual Meeting American Society for Microbiology, San Francisco, CA.

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Peer-Reviewed Conference Proceeding publications (other than abstracts):

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Received	Paper	
2012/07/08 1	12	Samantha T Parks, Yi Qu, Jim C Spain. The gene that encodes 4-hydroxy-3-nitrophenylacetate denitrase, the key enzymatic step in 3-nitrotyrosine degradation in Variovorax sp. JS669, Applied and Environmental Microbiology (06 2012)

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Books

Received Paper

TOTAL:

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Patents Awarded

Awards

Jim Spain. 2009 Proctor & Gamble Award in Applied and Environmental Microbiology, The American Society for Microbiology.

Second place, President's award for graduate student poster. Parks, S.T., Y. Qu, and J.C. Spain. (2009). Identification and Characterization of denA, a Novel HNPA Denitrase Integral to 3-Nitrotyrosine Degradation in Variovorax sp. JS669. Poster presented at the 95th Annual ASM Southeastern Branch Conference.

First place, President's award for graduate student presentation. Parks, S.T., Lee, S., and J.C. Spain. (2009). Biodegradation of 2-Nitropropylbenzene, a Synthetic Analog of 1-Nitro-2-phenylethane by Burkholderia sp. JS670. 95th Annual ASM Southeastern Branch Conference.

2nd Place, SAIC award, Best paper in environmental engieering by a student at Georgia Institute of Technology during 2009. Shin, K. A., and J. C. Spain. 2009. Biodegradation of diphenylamine by Burkholderia sp. strain JS667: pathway and evolutionary implications. Appl. Environ. Microbiol. 75:2694-270

Graduate Students

NAME	PERCENT_SUPPORTED	Discipline
Kurt, Zohre	0.25	
Parks, Samantha	0.75	
Qu, Yi	0.75	
Shin, Kwanghee	0.25	
FTE Equivalent:	**2.00**	
Total Number:	**4**	

Names of Post Doctorates

NAME	PERCENT_SUPPORTED
Payne, Rayford	1.00
Craven, Sarah	1.00
FTE Equivalent:	**2.00**
Total Number:	**2**

Names of Faculty Supported

NAME	PERCENT_SUPPORTED	National Academy Member
Spain, Jim C	0.12	
Nishino, Shirley	0.50	
FTE Equivalent:	**0.62**	
Total Number:	**2**	

Names of Under Graduate students supported

NAME	PERCENT_SUPPORTED
FTE Equivalent:	
Total Number:	

Student Metrics

This section only applies to graduating undergraduates supported by this agreement in this reporting period

The number of undergraduates funded by this agreement who graduated during this period:	0.00
The number of undergraduates funded by this agreement who graduated during this period with a degree in science, mathematics, engineering, or technology fields:	0.00
The number of undergraduates funded by your agreement who graduated during this period and will continue to pursue a graduate or Ph.D. degree in science, mathematics, engineering, or technology fields:	0.00
Number of graduating undergraduates who achieved a 3.5 GPA to 4.0 (4.0 max scale):	0.00
Number of graduating undergraduates funded by a DoD funded Center of Excellence grant for Education, Research and Engineering:	0.00
The number of undergraduates funded by your agreement who graduated during this period and intend to work for the Department of Defense	0.00
The number of undergraduates funded by your agreement who graduated during this period and will receive scholarships or fellowships for further studies in science, mathematics, engineering or technology fields:	0.00

Names of Personnel receiving masters degrees

NAME	
Kurt, Zohre	
Total Number:	**1**

Names of personnel receiving PHDs

NAME	
Parks, Samantha	
Qu, Yi	
Shin, Kwanghee	
Husserl, Johana	
Total Number:	**4**

Names of other research staff

NAME	PERCENT_SUPPORTED
FTE Equivalent:	
Total Number:	

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Inventions (DD882)

Scientific Progress

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Statement of the Problem

Within the DoD there is strong interest in development of green chemistry strategies for synthesis of explosives, novel biosensors for detection of explosives and chemical agents, and new biocatalysts for decontamination and demilitarization. The major bottleneck to application is our limited understanding of the metabolic diversity of biochemical systems that provide the basis for the technologies. With the advent of modern molecular biology and metagenomics it has become clear that all of the biochemistry described to date is only a fraction of the metabolic diversity to be found in nature. The tools for massive sequencing projects and bioinformatics are incredibly powerful for exploring metabolic diversity. The weakness of the approach, however, is that it is only possible to search for what is known (72). A combination of culture based approaches including selective enrichment combined with molecular tools is required to discover truly new biochemistry. The primary goal of this project was to reveal previously unexplored metabolic diversity among microbes that synthesize or degrade natural nitro compounds or natural acetylcholinesterase inhibitors. The hypothesis was that selective enrichment with natural chemical analogs could reveal metabolic diversity related to military chemicals. The ultimate payoff of the basic research is an enhanced understanding of natural metabolic diversity that will enable practical applications in synthesis, detection, and destruction of explosives. The insights about metabolic diversity are directly applicable to the discovery of new biomolecules for detection or destruction of explosives and chemical agents.

Background and significance

There is a growing awareness of the widespread synthesis of nitro organic compounds of natural origin in a variety of marine and terrestrial ecosystems (Fig. 1, (37, 52)). Some natural nitro compounds are produced by biochemical oxidation of the corresponding amino compounds (40). In plants nitration of a variety of aromatic compounds is catalyzed by the nitrogen dioxide radical produced from nitrite and hydrogen peroxide by the action of peroxidase enzymes (5, 62). Metmyoglobin and methemoglobin can catalyze similar reactions in animals to form a wide range of nitrated molecules (25).

Many other nitrated molecules are the result of direct nitration by peroxynitrite produced from the reaction between nitric oxide (NO) and superoxide (4). The discovery of the physiological role played by NO in a variety of systems was the basis for the Nobel Prize in Physiology or Medicine in 1998 (45). There is mounting evidence that nitration and denitration of tyrosine and other aromatic compounds in proteins play key roles in signaling and regulation. NO is formed in bacteria by both nitric oxide synthease (NOS) (1) and by nitrite reductase (10). In soil bacteria, particularly actinomycetes, NOS catalyzes reactions leading to the direct biochemical nitration of aromatic compounds including tryptophan and tyrosine during synthesis of antibiotics and

phytotoxins (6, 38). Bacterial NOS has been linked to tryptophan nitration for the production of the phytotoxin thaxtomin A (38). Finally, atmospheric pollution has been linked to the nitration of proteins in plant pollen (17).

While the list of natural nitro compounds is growing, the mechanism for their synthesis is often unknown, and even less is known about their biodegradation. It is clear, however, that such compounds do not accumulate in natural ecosystems. Based on a number of precedents it seems reasonable to assume that there must be specific catabolic pathways for their degradation, detoxification and recycling.

The mounting evidence of the wide array of nitro compounds and the substantial flux in the biosphere suggests that there is a previously unexplored metabolic diversity for synthesis and destruction of the compounds. For example, genes that encode nitroreductase enzymes are ubiquitous in biological systems. At least three are present in E. coli and similar numbers are present in all other bacteria. A recent search of the GenBank database revealed over 29,800 “nitroreductases” in the protein database, an increase of more than 26,000 in 5 years. The physiological roles of most of them are a complete mystery. A very large family of genes encodes enzymes related to “old yellow enzyme” and “diaphorases”, many of which are very effective nitroreductases (74), but their physiological roles and capabilities are also unknown (73).

One intriguing possibility is that the nitroreductases exist to degrade or detoxify nitro compounds produced by their own metabolism or by other organisms in their habitats. Such a scenario certainly applies in the case of antibiotic production and resistance in soil bacteria (11). The metabolic diversity involved in the production and cycling of nitro compounds might account for a substantial fraction of the unknown open reading frames in sequenced genomes.

The biological systems for production of the nitro compounds have considerable potential as a source of biocatalysts for green chemistry synthesis of commercially important nitro compounds. Conversely, the bacterial pathways for degradation of natural nitro compounds provide a rich source of genes that can be recruited for assembly of degradation pathways for synthetic nitro compounds released into the biosphere as explosives, pesticides, solvents and dyes. Indeed, the remarkably rapid evolution of catabolic pathways for synthetic nitroaromatic compounds that have only been in the biosphere for the past century argues strongly for a preexisting array of catabolic pathways that served as the basis for assembly of the novel pathways. Such a metabolic potential can be invoked to explain why many synthetic pesticides and antibiotics become ineffective after only a few years of use (50).

Many pesticides and the most toxic nerve agents act by irreversibly binding to acetylcholinesterase (AChE) (69), the enzyme that destroys the neurotransmitter, acetylcholine, after a signal is transmitted across the gap between neurons. The source of the resistance to new synthetic pesticides might lie in mechanisms that biota have developed to resist natural AChE inhibitors. There is a growing awareness of the remarkable array of natural AChE inhibitors produced by a wide variety of plants, animals, and microbes (28, 29). The situation with respect to the natural AChE inhibitors must be exactly analogous to that of the natural nitro compounds. The widespread production of the compounds in natural ecosystems requires an equally widespread metabolic capacity for the degradation of the molecules. That metabolic capacity might form the basis for strategies for detection and destruction of chemical agents

Organophosphorus hydrolase and the organophosphorus acid anhydrolase isolated from bacteria have provided the basis for the highly effective metabolic engineering strategies that have led to practical decontamination and force protection strategies (24). They are homologous to certain dipeptidases (8), but their physiological roles in their original bacterial hosts are unknown. Such hydrolases have been isolated from a variety of habitats by screening for their hydrolase activity or for the ability of the bacteria to grow on organophosphate pesticides (which are synthetic organic chemicals). What has not been attempted is a strategy for selective enrichment of bacteria able to use the natural analogs as growth substrates. Selection of such bacteria and elucidation of their biodegradation pathways could provide a remarkable source of novel enzymes for destruction or detection of synthetic analogs, including chemical agents.

The release of synthetic nitroaromatic compounds to the biosphere during the past century provided microbial populations with new sources of carbon, nitrogen, and electron acceptors, but new catabolic pathways had to evolve before the novel substrates could be exploited for growth. Pathways for degradation of novel compounds evolved through well-recognized processes of gene transfer, genetic mutation, and recombination and transposition (36) but the origins of the genes are mostly unknown. In some instances the pathways are poorly regulated and not very efficient, as if they have been assembled only recently. In some instances the bacteria that degrade explosives have only evolved the ability to use part of the molecule (71). It seems likely that some if not all of the pathways were assembled in soil bacteria by recruitment of portions of existing pathways for degradation of natural nitro compounds, but nothing is known about the possible links.

The purpose of this project was to explore the potentially much greater metabolic diversity of catabolic pathways for natural nitro compounds and AChE inhibitors. Our hypothesis was that the biochemistry is ancient, varied, widespread, and underappreciated. The metabolic diversity for degradation/destruction of natural compounds has very likely been the starting point for the recent and rapid evolution of pathways for degradation of synthetic chemicals.

A growing number of naturally occurring nitro compounds have been identified, but none consistently occur in high concentrations in the environment. Paradoxically, low detectable concentrations do not preclude the possibility of high flux in natural ecosystems. Recent discoveries suggest that, in some instances, the naturally occurring nitro compounds might have significant flux in the environment and the presence of such compounds has led to their exploitation by bacteria as growth substrates. For example, 4-nitroanisole-degrading bacteria were isolated from a mixture of farm and forest soils (65) that had no apparent prior exposure to 4-nitroanisole from anthropogenic sources. 4-Nitroanisole is produced by the basidiomycete, Lepista deimii (14), and it seems likely that the pathway for 4-nitroanisole degradation evolved in response to the presence of naturally occurring 4-nitroanisole in the soils. 4-Nitroanisole does not seem to accumulate in the environment, yet existence of a

well-developed pathway for its degradation suggests that there is a significant flux of the compound in the soil.
A similar situation exists for other naturally occurring nitro compounds including 3-nitrotyrosine (3NTyr) (48) and 3-nitropropionic acid (3NPA) (47). In addition, we have isolated a wide range of different bacteria able to grow on synthetic nitroaromatic compounds from contaminated soils. We have not found such bacteria in nearby uncontaminated soils, which clearly indicates that the detection of the specific metabolic pathways is a strong predictor of the presence and flux of the chemical.
3NTyr is particularly interesting because it is the predominant product of the interaction of reactive nitrogen species with proteins and peptides (35). It has been found at the site of every inflammatory response in mammals (31), and it might also have a crucial role in cell signalling (23, 43). A SciFinder search for “nitrotyrosine” reveals 5930 references about its synthesis and physiological role, but nothing was known about its subsequent metabolism until our recent work (48). We focused our initial experiments on 3NTyr because of its wide distribution and key role in animals and because we have discovered soil bacteria that use it as the sole source of carbon, nitrogen and energy (48).
Degradation of 3NTyr is catalyzed by a pathway (Fig. 2) that is inducible and distinct from the tyrosine degradation pathways (48). The ubiquity of 3NTyr in biological systems and the facile isolation of bacteria able to grow on it suggest that release of considerable quantities in the biosphere has led to the evolution and wide distribution of the pathway for degradation. Other nitro compounds in Fig. 1 can be readily detected when the organisms that produce them are studied in isolation, yet the compounds do not accumulate in the environment. It is highly likely that bacteria with appropriate catabolic pathways present in the same habitats account for the lack of accumulation. The overall objective of the research was to elucidate the mechanisms of metabolism of natural nitro compounds. We searched in a variety of ecosystems for bacteria that degrade different classes (nitroaromatic, nitroaliphatic, N-nitro, organophosphorus) of important natural compounds to determine whether the metabolism is based on recurring themes or tailored specifically to each molecule. Our purpose was to reveal the full range of metabolic potential for detection, synthesis and destruction of nitro compounds. The approach was subsequently applied to a natural organophosphate to determine whether the principles can be applied to other military unique chemicals.
Work performed
There were five specific goals of the research:
- Discovery of bacteria and determination of the biochemical mechanism(s) involved in the microbial metabolism of natural compounds beginning with 3NTyr followed by 3NPA.
  - Additional compounds were screened and added (5-nitroanthranilic acid (5NA), 1-nitro-2-phenylethane (NPE), chloramphenicol (Cam), N-nitroethylenediamine (NEDA) and cinnamoylphosphoramide (CPhos), etc.) or removed (pyrollnitrin, cyclopostin) from further study based on availability of the compounds or the results of preliminary enrichments.
- Determination of the molecular basis for the pathways by isolation of the genes and evaluation of their evolutionary origins
- Examination of the distribution of the catabolic pathways and determination of the extent to which the biochemistry is conserved across taxa
- Evaluation of the degree to which the ancient pathways for biodegradation of natural compounds provide the basis for rapid evolution of catabolic pathways for synthetic compounds of interest to DoD, including explosives, pesticides, and chemical agents
- Use of molecular information to design probes and primers to allow exploration of diverse habitats for the detection of the catabolic potential which will indicate the presence of synthetic pathways

Summary of the Most Important Results
At the start of our project we found literature reports of 210 natural nitro compounds (52) including analogs of nitrate ester, nitroaliphatic, nitroaromatic, nitramine and nitroheterocyclic explosives as well as several natural organophosphate compounds. In almost every case we attempted it was possible to isolate bacteria able to grow on the natural compounds, which means there are many enzymes evolved specifically for attack on the nitro and phospho groups.
We have established the biochemistry and molecular biology of the catabolic pathways for several of the molecules. The new enzymes and genes involved in the elimination of the key functional groups could not have been found by bioinformatics.
The story emerging from our findings with natural nitro compounds is that there is a vast and untapped metabolic diversity among the bacteria involved in synthesis and degradation of the millions of natural organic compounds. Such diversity can provide the basis for evolution of pathways for degradation or biosynthesis of new chemicals of interest to the DoD. The novel enzymes and genes also suggest an explanation for the existence of the daunting number of unknown and misannotated genes that make up over half the entries in the public databases (41, 55, 61, 66).
Many of the original tasks outlined in the proposal are complete while others have gone more slowly than expected. On the other hand, we have been able to pursue some unexpected opportunities such as the discovery of the first bacteria able to grow on nitroglycerin. Recently we isolated bacteria able to grow on DNAN, an insensitive explosive being phased in as a drop-in replacement for TNT.
Initial efforts were focused on extending our previous work with 3NTyr- and 3NPA-degrading bacteria as model systems to answer the above questions. We selected several other naturally occurring compounds to include in initial screens for bacteria that can grow on such compounds. The selection, based on similarity of the compounds to synthetic explosives and organophosphates, included NPE, a volatile phenyl-substituted nitroaliphatic compound produced by a variety of plants; 5NAA, a polar nitroaromatic compound produced by a variety of Streptomyces; Cam, a nitroaromatic antibiotic produced by streptomycetes; and NEDA, an N-nitro compound produced by Streptomyces that is an analog of the explosive

ethylenedinitramine (Haleite or N,N'-dinitroethylene diamine). Additional compounds were included in later screens. Selective enrichments in which the above natural compounds were individually provided as the sole carbon, nitrogen and energy source were started with locally collected soils as inocula.

Following the initial discovery of new metabolic pathways the emphasis of the project shifted to understanding the diversity and distribution of the catabolic genes and their relatedness to genes involved in the degradation of synthetic nitro compounds. This purpose was to extend the understanding of biochemical and metabolic diversity involved in degradation of nitro compounds, and to reveal the potential for the genes to be recruited for evolution of bacteria able to degrade explosives. We also established a collaboration with a synthetic chemistry group to obtain Cphos, a newly discovered natural organophosphate. Declining costs of next-gen sequencing enabled us to take advantage whole genome sequencing to explore the context of catabolic genes in organisms isolated for this project. The details of the specific compounds and metabolic pathways follow.

3-Nitrotyrosine

The pathway for degradation of 3-nitrotyrosine (3NTyr) was described in Burkholderia sp. JS165 and Variovorax paradoxus JS171 (48). Although never found in high concentrations in the environment, 3NTyr is widespread in the biosphere and is used as a marker for inflammation. Bacterial degradation of 3NTyr is initiated by an α-ketoglutarate-dependent deamination to the presumed intermediate 4-hydroxy-3-nitrophenylpyruvate (HNPP). Decarboxylation of HNPP yields 4-hydroxy-3-nitrophenylacetate (HNPA). The key reaction is the denitration of HNPA to homoprotocatechuate which is degraded by well-characterized central metabolic pathways. Both the initial deamination and the denitration of HNPA are specific for 3NTyr degradation. A gene (denA) that encodes an HNPA denitrase in V. paradoxus JS171 was cloned into E. coli and sequenced.

Differences in the biochemical characterization of HNPA denitrases from that of JS171, and the failure of denA-based primers to make a product from Burkholderia sp. JS165 suggested that denA represents only a portion of the genomic and biochemical variability involved in 3NTyr degradation even though all 3NTyr-degrading isolates tested used the degradation pathway found in the originally reported 3NTyr-degrading strains.

The PCR primers that amplified denA in JS171 amplified a similar product in a different Variovorax isolate, strain JS669. A denA deletion mutant was created in JS669 to confirm that the product of denA is involved in the denitration of HNPA. The mutant transforms 3NTyr more slowly than the wild-type, and has lost the ability to grow on HNPA (Fig. 3). The results indicate that denA is responsible for HNPA denitrase activity in the wildtype JS669.

The original degenerate denA primers identified denA genes in less than 25 percent of tested strains. New degenerate primers were designed to target conserved regions of denA in order to detect a broader range of 3NTyr-degrading bacteria. Tests of the new primers identified putative denA genes in some isolates that failed to yield products with the original primers. It appears that the new set of denA primers will facilitate detection of 3NTyr-degraders in environmental samples.

The amino acid sequences of DenA from Variovorax sp. JS669 and other bacteria with HNPA denitrase activity form a distinct group within the FAD monooxygenases (Fig. 4). Homologs from Bradyrhizobium and Bordetella when expressed in E.coli also catalyzed the denitration of HNPA. Thus HNPA denitrases are a group of novel enzymes which could be the source of the progenitor of enzymes that catalyze removal of nitro groups from synthetic nitroaromatic compounds and explosives. The work clearly establishes the function of a group of enzymes whose roles were previously unknown.

3-Nitropropionic acid

3-Nitropropionic acid (3NPA) is one of the most widespread of the naturally occurring nitroaliphatic compounds. As such, it served as a model compound for nitroaliphatic explosives such as FOX 7s in the early stages of this effort. It is structurally related to nitroethane, a nitroalkane manufacturing intermediate of mercury fulminate. It is a toxic component found in hundreds of legumes (27). Several enzymes have been described with catalytic activity towards 3NPA, but not in organisms that use 3NPA as a growth substrate. The most widespread of such enzymes are 2-nitropropane dioxygenases that were originally described in the fungus Williopsis saturnus variant mrakii (39) and have since been detected in many different organisms (over 2000 sequences were listed in GenBank in June, 2008) with the advent of whole genome sequencing. Despite the widespread occurrence, the physiological substrate was unknown at the start of this project.

3NPA-degrading bacteria were isolated from common garden soil (46). Analysis of partial 16S rRNA gene sequences identified two representative strains as Cupriavidus sp., designated JS190 and Pseudomonas sp., designated JS189. When strain JS190 was grown on 3NPA as the sole carbon, nitrogen and energy source, 70% of the initial nitrogen accumulated as nitrate and 2% accumulated as nitrite (Fig. 5).

The gene (pnoA) that encodes the key enzyme for removal of the nitro group was cloned and sequenced, and the enzyme was purified and partially characterized. Preliminary results indicated that the enzyme is a nitronate monooxygenase. BLAST analysis (3) of the amino acid sequence of PnoA identified the gene as a member of COG2070, a group of genes conserved across taxa and related to 2-nitropropane dioxygenase genes (Fig. 6) which were originally defined by flavoproteins purified from the yeasts Neurospora crassa and Williopsis saturnus mrakii for the ability to catalyze the transformation of 2-nitropropane. The early discovery resulted in application of the name 2-nitropropane dioxygenase to the family of enzymes, although the physiological substrate of the enzymes was never identified.

Many organisms contain multiple genes annotated as encoding 2-nitropropane dioxygenase. Despite the apparent widespread distribution of the COG, to date only proteins from 5 organisms have been purified and characterized to varying degrees. No evidence has been put forward that establishes 2-nitropropane as the physiological substrate of any of the enzymes included in COG2070. Very recently, the proposed a name change (19) from 2-nitropropane dioxygenase to nitronate monooxygenase with the new Enzyme Commission designation E.C.1.13.12.16., was approved based on recharacterization of purified proteins from

the yeasts, Williopsis saturnus variant mrakii and Neurospora crassa. PnoA is biochemically distinct from the well-characterized nitronate monooxygenases, with only ~ 20 - 25% amino acid identity to the biochemically characterized enzymes.
We cloned 3 different putative 2-nitropropane dioxygenases from Burkholderia phytofirmans PsJN and Pseudomonas aeruginosa PAO1 that were among the proteins most closely related to propionate nitronate monooxygenase in the BLAST results. Enzyme assays indicated that the encoded proteins are highly specialized for propionate 3-nitronate (Table 1). The specificity of the enzymes for the nitronate tautomer of 3NPA (Fig. 7), confirmed that P3N is the physiological substrate rather than 2-nitropropane.
Ha et al. crystallized a 2-nitropropane dioxygenase from P. aeruginosa PAO1 and analyzed and aligned the amino acid sequence with several closely related 2-nitropropane dioxygenases (21), but did not determine the physiological substrate of the enzyme. When the propionate-3-nitronate monooxygenases described here were added to the alignment, only 3 of the highly conserved residues interacting with FMN along with the His152 identified as the catalytic base were conserved (Fig. 8). The evidence suggests that propionate-3-nitronate monoxygenases form a separate cluster within COG2070 and that many of the proteins annotated as 2-nitropropane dioxygenases, now nitronate monooxygenases, are in fact specific propionate-3-nitronate monooxygenases. The argument is supported by the fact that 3NPA is likely to be much more widespread in nature than 2-nitropropane and other nitroalkanes. Many of the current annotations generated by genome sequencing projects are wrong (18) and the situation with nitronate monooxygenase seems to be another example of annotation based only on modest sequence similarity without functional information.
Several pnoA-containing strains were tested for the ability to grow on 3NPA. All strains containing pnoA, but not the E. coli control lacking the pnoA gene, grew on 3NPA as a nitrogen source but not always as a carbon source. The result suggests the lack of a complete degradation pathway or inability to regulate such a pathway in some strains. It is possible that in some bacteria the presence of propionate-3-nitronate monooxygenase might be a detoxification mechanism similar to the proposed function in fungi and plants. It is also a plausible mechanism of scavenging nitrogen. There may be a continuum of enzymes with functions from protection to growth represented in the diversity of genes in COG2070. The enzymes are clearly the most prevalent strategy for destruction of nitroaliphatic compounds in nature. In collaboration with biochemists from Georgia State University, we have established that P3N is the actual physiological substrate of the original yeast enzymes (16). P3N monooxygenase seems to serve as a detoxification mechanism in yeast that likely increases fitness during interspecies competition.
The work described above enabled us to obtain funding from COMBREX, a collaborative NIH- funded effort to establish the function of important, but misannotated and unknown bacterial genes in the public databases. We are leveraging the funding from COMBREX to establish the function of 10 related “2-nitropropane dioxygenases” with our partners from Georgia State University. The approach will support conclusions about the diversity of the enzymes and their applicability to compounds of interest to DoD.
1-Nitro-2-phenylethane
1-Nitro-2-phenylethane (NPE) is a naturally occurring, aromatic compound with a nitroaliphatic side chain that is produced by a variety of plants. Burkholderia sp. JS670 grows on NPE and 2-nitro-1-phenylpropane (NPB), a synthetic compound with a longer sidechain, using the compounds as the sole carbon, but not as a sole nitrogen source. Preliminary results indicated that a dioxygenase was involved in NPE degradation. Simultaneous induction experiments indicate that Burkholderia JS670 has similar degradation pathways for NPE and biphenyl. The result suggested that NPE is degraded via a pathway similar to dioxygenase-mediated toluene and biphenyl degradation upper pathway and a hydrolase-mediated lower pathway (Fig. 9). Following ring-opening, the original side chain is released from the ring-cleavage product as 3NPA. The dienoate fragment is further metabolized as a carbon source.
Mutagenesis experiments and qRT-PCR confirmed the activity of the NPE dioxygenase and the upregulation of both the NPE dioxygenase (74-fold) and the NPE hydrolase genes (230-fold), demonstrating that the putative NPE dioxygenase and hydrolase are catabolically important and necessary for the biodegradation of NPE (Fig. 10). Deletion of the NPE dioxygenase gene abolished growth on NPE. The NPE dioxygenase and hydrolase are distantly related to biphenyl dioxygenases and hydrolases.
There are many natural analogs of NPE and NPB. We used auxanography (51) to determine whether Burkholderia sp. JS670 and the NPB/NPE dioxygenase mutant can degrade various natural and synthetic analogs of NPE and NPB (Table 2). The wild type organism grows on many aromatic compounds 2 or more carbons in the sidechain while the mutant seems to be limited to 1 carbon sidechains. The results seem to reveal a common strategy not only for elimination of side chains containing nitro groups, but also other structures that would be bottlenecks to catabolic pathways.
Diphenylamine
Diphenylamine (DPA) is a naturally occurring phytocompound that is widely used as a stabilizer for explosives and as a precursor of dyes, pesticides, pharmaceuticals, and photographic chemicals. DPA is also formed as a byproduct during the manufacture of aniline. DPA and its nitrated derivatives are common contaminants at munitions-contaminated sites as well as at manufacturing sites. DPA is biodegraded under both aerobic and anoxic conditions but the mechanisms and the organisms responsible for the biodegradation were unknown.
Burkholderia sp. strain JS667 and Ralstonia sp. strain JS668 were isolated by selective enrichment from sediment from a DPA-contaminated stream (68). The isolates grew aerobically with DPA as the sole carbon, nitrogen, and energy source. During induction on DPA, stoichiometric amounts of aniline accumulated and then disappeared, which suggested that aniline is on the DPA degradation pathway. A 40 kb fragment containing the genes that encode the enzymes that catalyze the initial

steps in DPA degradation was cloned from the genomic DNA of strain JS667. The E. coli clone catalyzed stoichiometric transformation of DPA to aniline and catechol. Transposon mutagenesis and the amino acid sequence similarity of putative open reading frames to those of well characterized dioxygenases support the conclusion that the initial reaction in DPA degradation is catalyzed by a multi-component ring-hydroxylating dioxygenase.

Genes that encode the complete aniline degradation pathway were found 12 kb downstream of the genes that encode the initial DPA dioxygenase. Reverse transcriptional PCR analysis confirmed the expression of each of the relevant dioxygenases in DPA grown cells of JS667. The results indicate that DPA is converted to aniline and catechol via angular dioxygenation and spontaneous rearomatization. Aniline and catechol are further biodegraded by the well established aniline degradation pathway. Genes that encode the complete DPA degradation pathway are on a 31 kb DNA segment. The sequence similarity and the gene organization suggest that the DPA degradation pathway evolved by recruitment of two gene clusters that encode DPA dioxygenase and an aniline degradation pathway (Fig. 11).

The organization of the genes that encode the DPA dioxygenase enzyme system is similar, but not identical to that of the well conserved car operon for degradation of carbazole, a natural compound structurally related to DPA. carC catalyzes the hydrolysis of the meta-cleavage products of 2'-aminobiphenyl-2,3-diol to anthranilate and 2-hydroxypenta-2,4-dienoate. The hydrolysis is not necessary in DPA degradation and carC is lacking in JS667. The presence of a truncated carBb in JS667, strongly suggests that the gene clusters involved in the initial steps in the DPA degradation pathway evolved by recruitment of the terminal dioxygenase gene from the carbazole degradation pathway (Fig. 12).

Recruitment of genes encoding DPA dioxygenase would be sufficient to allow common aniline degrading bacteria to grow with DPA as a sole source of carbon, nitrogen, and energy. The hypothesis is supported by several lines of evidence: the differences in G plus C content between the gene clusters that encode DPA dioxygenase and the aniline degradation components, several transposon remnants between the gene clusters, and that the aniline degradation operon is highly organized without superfluous genetic material relative to the DPA dioxygenase operon (Fig 11). The immature organization of the DPA operon seems to contradict the expectation that the genes encoding the degradation pathways of naturally occurring compounds are linked in operons without superfluous genetic material. It seems reasonable to speculate that the system was assembled at the field site in response to contamination by DPA. The genes of the assembled pathway appear to have been recruited from carbazole and aniline degradation pathways. Such assembly of degradative pathways in response to contamination has been observed in a chlorobenzene-degrading organism (44).

Bacteria able to grow on aniline, DPA, or carbazole as sole carbon, nitrogen, and energy source were isolated by selective enrichment from the same soil that yielded the original DPA-degrading strains. All of the DPA-degrading bacteria were able to grow on DPA and aniline, but not on carbazole. Neither carbazole nor aniline-degrading bacteria could grow on DPA.

PCR amplification was carried out with the primers based on nucleotide sequences of dpaAa from JS667. The internal region (800 bp) of dpaAa was amplified in the all the DPA degraders, but not in the carbazole degraders. The nucleotide sequences of the amplicons from the DPA-degrading strains were identical. The presence of identical DPA dioxygenase genes among distantly related DPA-degrading bacteria is indicative of horizontal gene transfer of the initial dioxygenase gene.

Similarly, a highly conserved 371 bp region of the aniline dioxygenase gene was amplified from the DPA- and aniline-degrading bacteria, but not from the carbazole-degrading isolates. The results indicate that DPA and aniline degraders have aniline dioxygenase genes. When PCR primers based on the gene cluster that encodes aniline dioxygenase in JS667 were used to search for homologs, identical DNA bands were observed in DPA degraders and one of the aniline degraders. The results indicate that they share highly similar aniline dioxygenase genes. The results are consistent with the proposal that genes from carbazole and aniline biodegradation pathways are the source of the genes that compose the DPA degradation pathway.

5-Nitroanthranilic acid

5-Nitroanthranilic acid (5-NAA), a natural nitroaniline, is produced by Streptomyces scabies. 5NAA can also be the starting material for synthesis of various nitroaromatic compounds and dyes. The introduction of the amino group into nitroaromatic compounds can make the resulting compounds more thermally stable. In the following series of aminonitrobenzene explosives, the thermodynamic stability increases from mono-amino-2,4,6-trinitrobenzene (MATB), to 1,3-diamino-2,4,6-trinitrobenzene (DATB), and 1,3,5-triamino-2,4,6-trinitrobenzene (TATB). Biodegradation pathways of nitro aromatic compounds and anilines are well documented, but nothing is known about that of nitroanilines.

Selective enrichment with 5NAA as the sole carbon and nitrogen source yielded Bradyrhizobium sp. JS329 (56). The first step in 5NAA degradation is deamination to form stoichiometric amounts of 5-nitrosalicylic acid (5NSA). Dialyzed and undialyzed cell extracts catalyze the initial reaction at almost the same rates. The result suggests that the deaminase requires no dialyzable cofactors such as flavin, metals, or electron donors. The further transformation of 5NSA doesn’t require FAD, FMN, or extra electron donor, but does require Fe2+. Transformation of 5NSA is inhibited by the flavin enzyme inhibitor diphenyliodonium, by the cytochrome P450 inhibitor metapyrone, and by chloramphenicol, and EDTA.

A genomic library of JS329 was screened for clones with the ability to transform 5NAA. Genes that encoded enzymes involved in 5NAA or 5NSA degradation were inactivated by insertion of the Tn5 transposon and the flanking regions were sequenced. The putative 5NAA deaminase is deeply branched and only distantly (34% amino acid identity) related to an uncharacterized M20/M25/M40 family peptidase from Hyphomonas neptunium ATCC 15444. 5NAA deaminase is the first hydrolase that removes an amino group from a benzene ring. When the gene that encodes 5NAA deaminase was cloned and over-expressed, enzyme assays revealed that the deaminase is specific for 5NAA. It did not attack anthranilate, 3-nitrobenzoate, 4-nitroaniline, 5-hydroxyanthranilic acid, or 4-nitroanthranilic acid, which indicates that the -NO2 and COOH substituents are essential for the deamination activity.

5NSA 1,2-dioxygenase is distantly related to gentisate 1,2-dioxygenase from Oligotropha carboxidovorans OM5 (29% amino acid identity) and salicylate 1,2-dioxygenase from Pseudaminobacter salicylatoxidans (22% amino acid identity). The enzymes share a conserved histidine pair serving to anchor Fe2+ and a conserved domain. 5NSA dioxygenase is active against salicylate, 5-chlorosalicylate, and 5-bromosalicylate; and inhibited by iron chelators. The properties are similar to those of salicylate 1,2-dioxygenase (26), but salicylate 1,2-dioxygenase does not attack 5NSA (26). It is clear that 5NSA dioxygenase is specialized for the nitro-substituted compound.

Fumarate and pyruvate were products of enzyme assays that consumed 5NSA without additional cofactors. Two alternatives can account for the observed conversion: formation of a lactone accompanied by the removal of the nitro group, followed by hydrolytic ring cleavage to produce maleylpyruvate; or formation of a lactone without the release of nitrite, which is then hydrolyzed to maleylpyruvate with the removal of nitro group.

To identify the gene involved in the removal of the nitro group from the nitroaliphatic compound, the 5NAA fosmid DNA was sequenced, and one large fragment was annotated (58). Potential enzymes involved in the biodegradation of 5NAA are highlighted (Fig. 13). The proposed pathway is consistent with one reported for 5-halosalicylate (26). This study provides the first example for the denitration mechanism for the ring fission products, and provides the first clear evidence for the biodegradation of amino-nitroaromatic compounds.

5NAA deaminase (NaaA, Fig. 14) is the only hydrolase known to remove an amino group attached to the benzene ring, To determine biochemical properties of the enzyme, the gene was cloned in E. coli, and the protein was overexpressed. NaaA was purified by His-tag affinity chromatography. A collaboration was established with a lab specializing in structural biology and low resolution X-ray analysis of NaaA was obtained, but efforts are continuing to optimize the conditions for crystallization so that high resolution X-rays may be obtained. Potential substrates will be predicted by docking an array of nitroaniline explosives to the enzyme structure. Understanding of the substrate binding pocket will allow design of mutant enzymes that can bind explosive analogs of 5NAA.

4-Nitroaniline

The presence of the hydrolytic deaminase in the degradation pathway for 5NAA raised the question of whether the same mechanism can be applied to degradation of synthetic nitroanilines. 4-Nitroaniline (4NA) is a widely used synthetic intermediate that was previously reported to be biodegraded by an unknown mechanism. 4NA is an intermediate in the synthesis of dyes, antioxidants, pharmaceuticals, corrosion inhibitors and is very harmful to aquatic organisms (20, 64). It is structurally similar to natural compounds such as nitroanthranilic acids and synthetic explosives like N-methyl-4-nitroaniline (MNA), 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) that consist of nitro and amine groups attached to an aromatic ring. The multiply substituted aromatic ring makes such molecules hard to biodegrade.

Several studies related to 4NA biodegradation have been published over the years (60, 63, 76), but the complete biodegradation pathway of the 4NA remains to be established. Our objective was to rigorously establish the degradation mechanism, to determine whether the mechanism can be applied to explosives, and to discover the key genes and enzymes of 4NA biodegradation. Determination of the initial attack on 4-nitroaniline will provide insight about the effect of the carboxyl and nitro groups on the strategy for deamination.

Rhodococcus sp. strain JS360, capable of using 4NA as sole carbon and nitrogen source, was isolated from 4NA-contaminated soil. Simultaneous induction assays were used to infer possible pathway intermediates. During growth on 4NA nitrite accumulation is stoichiometric whereas only half of the theoretical ammonia accumulates, indicating that the ammonia is consumed for growth (Fig. 15). Oxygen uptake was stimulated by 4-aminophenol and 4-aminocatechol, but not 4-nitrocatechol, suggesting that the nitro group is released before the amino group. The chlorinated analog of 4NA, 4-chloroaniline also stimulated oxygen uptake. The result suggests that the enzyme that attacks the nitro group might also attack chloro substituents. 4NA-grown cells provided with 4-chloroaniline released stoichiometric amounts of chloride, suggesting that the first enzyme in the pathway is capable of attack on both chloro and nitro groups. Flavoproteins of the TC-FDM family are well known to be active with both chloro and nitro-substituted substrates (53).

The genome of JS360 was sequenced with the Illumina HiSeq2000 platform after a screen of the clone library failed to detect the gene responsible for the activity. The genome was annotated and primers were designed based on possible flavoprotein monooxygenases encoded in the genome. Genes encoding enzymes that are closely related to flavoproteins or to monooxygenases were cloned into E. coli. Preliminary tests indicated that an enzyme closely related to pentachlorobenzene monooxygenase initiates 4NA biodegradation. Analysis of the products of the enzymatic reaction is underway. The remaining clones will be tested in the same way to determine further steps and enzymes in the catabolic pathway (Fig. 16).

2-Nitroimidazole

2,4-Dinitroimidazole, is a powerful insensitive high explosive. 3-Nitro-1,2,4-triazol-5-one (NTO) is a related insensitive explosive that is currently emerging as a replacement for TNT and RDX. 2-Nitroimidazole (2NI), also known as azomycin, is a natural analog of 2,4-dinitroimidazole and NTO that is produced by actinomycetes and pseudomonads. 2NI inhibits the growth of bacteria, protozoa and a variety of anaerobic bacteria. Elucidation of the biodegradation pathway of 2NI was pursued to gain insight into potential mechanisms for dinitroimidazole biodegradation and for enzymatic removal of nitro groups from heterocycles in general.

Mycobacterium sp. JS330 was enriched from soil with 2NI provided as the sole carbon and nitrogen and energy source. Cultures grown on 2NI released stoichiometric amounts of nitrite and imidazol-2-one was detected in the culture medium (Fig. 17). Mycobacterium JS330 also grows on imidazol-2-one as the sole carbon, nitrogen and energy source, which suggests that

degradation 2NI is initiated by denitration to form imidazol-2-one.
The distribution of 2NI-degrading bacteria was determined in soil from a wide range of habitats from around US. Degradation of 2NI, accompanied by the accumulation of nitrite, was observed in 11 out of 13 samples. Six isolates obtained from the enrichments grew on 2-nitroimidazole or imidazol-2-one as the carbon and nitrogen source with the release of nitrite from 2NI. The isolates were most closely related to Mycobacterium brisbanense, Rhizobium sp., Wautersia sp., and Phyllobacterium myrsinacearum. Widespread ability to degrade 2NI indicates a substantial flux of 2NI in natural ecosystems and suggests an important ecological role of 2NI.
A fosmid that catalyzed the transformation of 2NI to nitrite and imidazol-2-one was sequenced. The gene that encodes 2NI nitrohydrolase (nnhA) was identified (Fig. 18) and the enzyme was characterized. 2NI nitrohydrolase catalyzes denitration of benznidazole but not 3- or 4-nitro substituted imidazoles, suggesting that the enzyme is specific for 2-nitro functional groups. The gene that encodes 2NI nitrohydrolase confers resistance to 2NI to E. coli. The finding reveals a new drug resistance mechanism in soil bacteria prior to its appearance in pathogens (2, 11, 12).
NnhA was purified by His-tag affinity chromatography. No added cofactors are required for activity of the purified protein. Conditions for crystallization of the enzyme are currently being optimized to enable x-ray crystallography. As the reaction catalyzed by the enzyme has not been previously described, it has been submitted to the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology (Enzyme Commission) for classification.
N-Nitroethylenediamine
N-Nitroethylenediamine (NEDA), is a natural nitramine compound, produced by the mushroom Agaricus silvaticus. NEDA is an analog of the insensitive high explosive ethylenedinitramine (EDNA or haleite, Fig. 19). EDNA can be either a primary explosive in detonators, as a booster charge, or as a secondary explosive. The N-nitro group found in NEDA is also a key component of RDX, HMX and other nitramine explosives. Determination of the unknown NEDA degradation pathway will provide insight to enable understanding of the metabolism of a wide range of nitramine explosives.
NEDA-degrading bacteria were isolated from soil for their ability to grow on NEDA as the carbon and nitrogen source (Fig. 20). It is clear that the isolates eliminate the nitro group as nitrite by an unknown mechanism. The genome of one of the NEDA-degrading isolates was sequenced. Analysis of the sequence will reveal whether a cytochrome P450 enzyme, similar to the XplA enzyme for RDX degradation (67) is involved. If similar genes are discovered, they will be examined to determine whether they are the progenitors of XplA. The biodegradation pathway will be studied using both biochemical and molecular biological approaches. The enzymes involved will be screened for the ability to denitrate nitramine explosives.
8- Nitroguanine
8-Nitroguanine (8NG) is a naturally occurring marker of DNA nitration. Nitroguanine is an analog of the explosive, 2,4-dinitroimidazole. The production of 8NG in biological systems is well understood, but nothing is known about its biodegradation. Because the subsequent metabolism and fate of the molecule in humans is a mystery, elucidation of the microbial degradation pathway could provide insight relevant to the biochemistry and toxicology. We synthesized 8NG and set up selective enrichments for bacteria able to use it as a growth source. We obtained an Pseudomonas putida isolate from soil that degrades 8NG under aerobic conditions.
The isolate grows well on guanine, but 8NG is toxic and is only partially degraded with stoichiometric release of nitrite. Experiments designed to determine the effects of 8NG on the cells indicate that 8NG inhibits cell growth irreversibly but no toxic metabolites accumulate in the medium. Various resins were tested for the ability to reduce the 8NG concentration in solution. Resin IRA 743 efficiently reduced the 8NG concentration but did not relieve the inhibition. Guanine supports growth of the bacteria but xanthine oxidase, which catalyzes the first step in the guanine degradation pathway, did not attack 8NG. We are currently determing the initial step in the 8NG degradation pathway and the source of inhibition during growth on 8NG alone.
Chloramphenicol
Chloramphenicol, an antibiotic compound produced by Streptomyces, is one of the best known naturally occurring nitroaromatic compounds. Despite much research dedicated to mechanisms of chloramphenicol resistance, the mechanisms for complete degradation of the molecule remains a mystery. Lingens et al. proposed a catabolic pathway, based solely on isolation and identification of metabolites (42), that has not been substantiated by biochemical or genetic evidence and in light of recent discoveries about degradation of synthetic nitroaromatic compounds, the previously proposed pathways warranted reexamination. Catabolic pathways that have evolved in soil bacteria to detoxify or exploit natural antibiotics represent a source of metabolic diversity. Genes that evolved for biodegradation additionally serve as a reservoir of antibiotic resistance determinants that may be transferred to pathogenic bacteria (12, 13).
We isolated Nocardia JS674 from soil based on its ability to subsist on chloramphenicol as a sole carbon and nitrogen source. Biochemical evidence suggests that the lower pathway for chloramphenicol degradation proceeds through 4-nitrobenzoate, and involves a partially reductive pathway that generates a hydroxylaminobenzoate intermediate. Enzyme assays confirm activity of an inducible 4-nitrobenzoate reductase and 4-hydroxylaminobenzoate lyase that are active when JS674 is grown on chloramphenicol (Table 3). The pathway is not induced when cells are grown on glucose or pyruvate.
The genome of JS674 was sequenced on the Illumina HiSeq2000 platform. Genes involved in the lower pathway for chloramphenicol degradation, including a 4-nitrobenzoate reductase and 4-hydroxylaminobenzoate lyase, were identified. The predicted enzymes from JS674 show high sequence similarity to characterized 4-nitrobenzoate reductase and 4-hydroxylaminobenzoate lyase enzymes from Ralstonia pickettii YH105 (48% and 60% similarity, respectively) and from Pseudomonas putida TW3 (44% and 57%, respectively) (30, 75). The enzymes involved in chloramphenicol degradation may be evolutionary precursors to those previously elucidated for related xenobiotic compounds such as 4-nitrotoluene (22), an

intermediate in the production of TNT

Nitroglycerin

Nitroglycerin (NG, GTN) is the best known of the many and varied nitrate ester explosives including ethylene glycol dinitrate (EGDN), diethylene glycol dinitrate (DEGN), pentaerythritol tetranitrate (PETN), and nitrocellulose (NC). Recently, natural nitrate esters have been discovered in marine and freshwater systems. The alcyopterosins (49), the first recognized natural nitrate esters, are produced by soft corals, and the alkyl nitrates, particularly methyl nitrate and ethyl nitrate, most likely have a microbial origin (9, 70). The discovery of naturally occurring nitrate esters raises the possibility that some enzymes have evolved specifically for the catalysis of nitrate esters, but nothing is known about biodegradation of the natural nitrate esters. Until recently, no microbes have been isolated that can grow on nitrate ester explosives. Previous research has shown that flavoproteins from bacteria and yeast can partially denitrate PETN and NG. In the case of NG, the endproducts are various mixtures of mono- and dinitroglycerins (MNG). In collaboration with Joe Hughes at Georgia Tech we isolated Arthrobacter sp. JBH1 that grows on NG (Fig. 21, (33)). We identified the flavoprotein that catalyzes the specific conversion of NG to 1MNG without accumulation of the dead end metabolite 2MNG. We sequenced the genome of strain JBH1 and used transposon mutagenesis to establish the identity of the key enzyme responsible for the further degradation of 1MNG (32). The enzyme is closely related to glycerol kinase and seems to phosphorylate MNG. The phosphorylation enables the subsequent utilization and complete degradation of MNG by an unknown mechanism (Fig. 22). The discovery of the key enzymes sets the stage for determining the biochemical properties and substrate ranges of the enzymes and how the catabolic pathway evolved. The NG degradation pathway seems to be in the early stages of evolution with enzymes that are not optimized and not regulated. The system is thus well suited for advancing our understanding about how such pathways evolve in response to the appearance of new synthetic chemicals by recruitment of genes from existing pathways.

2,4-Dinitroanisole

The DoD has recently begun the production of insensitive explosives including IMX-101, IMX-104 and PAX-21. The key component is 2,4-dinitroanisole (DNAN) a drop-in replacement for TNT. Although DNAN is not known to be a naturally occurring nitroaromatic compound, it is similar to 4-nitroanisole, a natural nitroaromatic compound produced by fungi and 2,4-dinitrophenol (24DNP) which is produced in the atmosphere by nitration of 2-nitrophenol. The catabolic pathways are known for both of the natural analogs and it seems likely that the biodegradation pathway for DNAN evolved by recruitment and modification of the genes that encode degradation of the natural analogs that have been in the biosphere for a very long time. We have recently isolated bacteria from Holston AAP that have the ability to grow on both DNAN and 24DNP, apparently by similar mechanisms. When the isolates are grown on DNAN 24DNP transiently accumulates in the medium (Fig. 23). Subsequent degradation of 24DNP leads to the release of the nitro groups as nitrite. The results are the first indication that DNAN can be biodegraded completely and they strongly suggest that the pathway is an extension of the 24DNP degradation pathway. Previous studies reported the accumulation of a dead-end metabolite under anaerobic conditions (54). Our hypothesis is that recruitment of a gene encoding a demethylase is necessary and sufficient for extending the pathway for degradation of 24DNP to enable degradation of the new explosive. The discovery of naturally occurring bacteria that degrade DNAN will provide the basis for prediction of biodegradation of the new explosive in the environment.

(S)-Cinnamoylphosphoramide

Because we were unable to obtain sufficient quantities of the natural organophosphates originally proposed we worked with the newly discovered cinnamoylphosphoramide (CPhos), an organophosphate isolated from a tunicate-associated Streptomyces (59). When CPhos was provided as a sole carbon, phosphorus, and nitrogen source in enrichments from seawater, tunicates, tunicate-associated fauna, and sponges, CPhos disappeared from the enrichments with the transient appearance of more polar metabolites. LC-MS analysis identified the major metabolite with mass of 179 as p-hydroxycinnamic acid methyl ester (HCAME), indicative of a loss of $CH_3PO_2NH_2$ (phosphoramidic acid methyl ester, Fig. 24).

Authentic HCAME was rapidly degraded by the enrichments when either CPhos or methamidophos, an organophosphate insecticide, and close structural analog to phosphoramidic acid methyl ester, was provided as the phosphorus and nitrogen sources (Fig. 25). In the absence of methamidophos or CPhos, HCAME was not degraded. The results indicate that methamidophos can replace phosphoramidic acid methyl ester as the nitrogen and phosphorus source. Neither phosphoramidic acid methyl ester nor CPhos are required to induce the degradation of methamidophos. The slightly faster degradation of CPhos in the presence of methamidophos might indicate that nitrogen is limiting when CPhos is the sole carbon, nitrogen, and phosphorus source.

Labrenzia sp. JS676 and Erythrobacter sp. JS677 were isolated from agar plates made with seawater containing Cphos as the sole growth substrate. Both isolates degrade HCAME (as sole carbon source) when provided with methamidophos (sole phosphorus and nitrogen source) or demeton-S-methyl (sole phosphorus source). The growth of the bacteria using methamidophos or demeton-S-methyl as phosphorus/nitrogen source indicates that the isolates possess enzymes that allows mineralization and subsequent assimilation of organophosphates (Fig 26). The isolates clearly contain a new hydrolase that has evolved to cleave the natural organophosphate. The ability to degrade methamidophos and demeton-S-methyl suggests that the OP hydrolase has a broad substrate specificity.

The genomes of both isolates were sequenced on the Illumina HiSeq2000 platform. Preliminary assembly and annotation revealed the presence of numerous alkylphosphonate (phn) genes in both isolates. No alkylphosphonate genes are present in the 2 public Erythrobacter genomes. Alkylphosphonate utilization is a secondary source of phosphorus for many bacteria that is suppressed in the presence of inorganic phosphate. There is indirect evidence that alkylphosphonate enzymes can attack ester-linked organophosphate nerve agents. The phnCDEFGHIJKLMNOP operon from E. Coli (7) consists of the

membrane-bound C-P lyase (phnGHIJKLM), transporter proteins (phnCDE) and several accessory and regulatory proteins. phnE in E. coli K-12 strains requires a specific 8 base pair deletion to activate the gene and allow expression of the phn operon (34). When phnE with the specific deletion was cloned into E. coli JA221, the strain was able to use diisopropylfluorophosphate as a phosphorus source (15). Diisopropylfluorophosphatase is the formal name of OPAA, one of the two widely studied enzymes that attack nerve agents.

Conclusions

The overall objective of elucidating the mechanisms of metabolism of natural compounds related to DoD compounds of interest has been accomplished. In almost every case that we studied, we were able to isolate organisms that could grow on the naturally occurring compound. In the cases where the natural compound is widespread, such as for 3NTyr and 3NPA, isolates able to degrade the compounds are also widely distributed and readily obtained. Other isolates were only found in specialized environments when the natural product was reported from only a limited number of sources. Such was the case with 2-nitroimidazole and and 5-nitroanthranilic acid.

Novel biochemistry for the biodegradation of the compounds was identified in most instances which was in turn linked to previously unrealized gene clusters. The work with 3NPA lead to solving the 50 year-old mystery of the true physiological substrate and purpose of 2-nitropropane dioxygenase. In the case of DPA, we were able to identify the genetic components that combined to form the DPA degradation operon. The results have borne out our initial hypothesis that there is untapped metabolic diversity for degradation of natural compounds.

Ongoing Work

Several aspects of the project are still ongoing. Crystallization of 5NAA deaminase and 2NI nitrohydrolase for X-ray analysis will enable detailed characterization of the enzymes. Studies on the 4NA degradation pathway are continuing with cloning of genes likely to be involved in the pathway and analysis of their products for activity. Similarly we are analyzing the products of genes cloned from chloramphenicol- and nitroglycerin-degrading isolates in order to clearly establish their roles in the biodegradation pathways.

Recommendation

Technology Transfer

Final Report
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Metabolic Diversity for Degradation, Detection, and Synthesis of Nitro Compounds and Toxins

Shirley F. Nishino
Jim C. Spain, P.I.
Sarah H. Craven
Johana Husserl
Zohre Kurt
Samantha T. Parks
Rayford B. Payne
Yi Qu
Kwanghee A. Shin

Georgia Institute of Technology, School of Civil and Environmental Engineering
Atlanta, GA 30332-0512

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Statement of the Problem

Within the DoD there is strong interest in development of green chemistry strategies for synthesis of explosives, novel biosensors for detection of explosives and chemical agents, and new biocatalysts for decontamination and demilitarization. The major bottleneck to application is our limited understanding of the metabolic diversity of biochemical systems that provide the basis for the technologies. With the advent of modern molecular biology and metagenomics it has become clear that all of the biochemistry described to date is only a fraction of the metabolic diversity to be found in nature. The tools for massive sequencing projects and bioinformatics are incredibly powerful for exploring metabolic diversity. The weakness of the approach, however, is that it is only possible to search for what is known (72). A combination of culture based approaches including selective enrichment combined with molecular tools is required to discover truly new biochemistry. The primary goal of this project was to reveal previously unexplored metabolic diversity among microbes that synthesize or degrade natural nitro compounds or natural acetylcholinesterase inhibitors. The hypothesis was that selective enrichment with natural chemical analogs could reveal metabolic diversity related to military chemicals. The ultimate payoff of the basic research is an enhanced understanding of natural metabolic diversity that will enable practical applications in synthesis, detection, and destruction of explosives. The insights about metabolic diversity are directly applicable to the discovery of new biomolecules for detection or destruction of explosives and chemical agents.

Background and significance

There is a growing awareness of the widespread synthesis of nitro organic compounds of natural origin in a variety of marine and terrestrial ecosystems (Fig. 1, (37, 52)). Some natural nitro compounds are produced by biochemical oxidation of the corresponding amino compounds (40). In plants nitration of a variety of aromatic compounds is catalyzed by the nitrogen dioxide radical produced from nitrite and hydrogen peroxide by the action of peroxidase enzymes (5, 62). Metmyoglobin and methemoglobin can catalyze similar reactions in animals to form a wide range of nitrated molecules (25).

Many other nitrated molecules are the result of direct nitration by peroxynitrite produced from the reaction between nitric oxide (NO) and superoxide (4). The discovery of the physiological role played by NO in a variety of systems was the basis for the Nobel Prize in Physiology or Medicine in 1998 (45). There is mounting evidence that nitration and denitration of tyrosine and other aromatic compounds in proteins play key roles in signaling and regulation. NO is formed in bacteria by both nitric oxide synthease (NOS) (1) and by nitrite reductase (10). In soil bacteria, particularly actinomycetes, NOS catalyzes reactions leading to the direct biochemical nitration of aromatic compounds including tryptophan and tyrosine during synthesis of antibiotics and phytotoxins (6, 38). Bacterial NOS has been linked to tryptophan nitration for the production of the phytotoxin thaxtomin A (38). Finally, atmospheric pollution has been linked to the nitration of proteins in plant pollen (17).

While the list of natural nitro compounds is growing, the mechanism for their synthesis is often unknown, and even less is known about their biodegradation. It is clear, however, that such compounds do not accumulate in natural ecosystems. Based on a number of precedents it seems reasonable to assume that there must be specific catabolic pathways for their degradation, detoxification and recycling.

The mounting evidence of the wide array of nitro compounds and the substantial flux in the biosphere suggests that there is a previously unexplored metabolic diversity for synthesis and destruction of the compounds. For example, genes that encode nitroreductase enzymes are ubiquitous in biological systems. At least three are present in *E. coli* and similar numbers are present in all other bacteria. A recent search of the GenBank database revealed over 29,800 "nitroreductases" in the protein database, an increase of more than 26,000 in 5 years. The physiological roles of most of them are a complete mystery. A very large family of genes encodes enzymes related to "old yellow enzyme" and "diaphorases", many of which are very effective nitroreductases (74), but their physiological roles and capabilities are also unknown (73).

One intriguing possibility is that the nitroreductases exist to degrade or detoxify nitro compounds produced by their own metabolism or by other organisms in their habitats. Such a scenario certainly applies in the case of antibiotic production and resistance in soil bacteria (11). The metabolic diversity involved in the production and cycling of nitro compounds might account for a substantial fraction of the unknown open reading frames in sequenced genomes.

The biological systems for production of the nitro compounds have considerable potential as a source of biocatalysts for green chemistry synthesis of commercially important nitro compounds. Conversely, the bacterial pathways for degradation of natural nitro compounds provide a rich source of genes that can be recruited for assembly of degradation pathways for synthetic nitro compounds released into the biosphere as explosives, pesticides, solvents and dyes. Indeed, the remarkably rapid evolution of catabolic pathways for synthetic nitroaromatic compounds that have only been in the biosphere for the past century argues strongly for a preexisting array of catabolic pathways that served as the basis for assembly of the novel pathways. Such a metabolic potential can be invoked to explain why many synthetic pesticides and antibiotics become ineffective after only a few years of use (50).

Many pesticides and the most toxic nerve agents act by irreversibly binding to acetylcholinesterase (AChE) (69), the enzyme that destroys the neurotransmitter, acetylcholine, after a signal is transmitted across the gap between neurons. The source of the resistance to new synthetic pesticides might lie in mechanisms that biota have developed to resist natural AChE inhibitors. There is a growing awareness of the remarkable array of natural AChE inhibitors produced by a wide variety of plants, animals, and microbes (28, 29). The situation with respect to the natural AChE inhibitors must be exactly analogous to that of the natural nitro compounds. The widespread production of the compounds in natural ecosystems requires an equally widespread metabolic capacity for the degradation of the molecules. That metabolic capacity might form the basis for strategies for detection and destruction of chemical agents

Organophosphorus hydrolase and the organophosphorus acid anhydrolase isolated from bacteria have provided the basis for the highly effective metabolic engineering strategies that have led to practical decontamination and force protection strategies (24). They are homologous to certain dipeptidases (8), but their physiological roles in their original bacterial hosts are unknown. Such hydrolases have been isolated from a variety of habitats by screening for their hydrolase activity or for the ability of the bacteria to grow on organophosphate pesticides (which are synthetic organic chemicals). What has not been attempted is a strategy for selective enrichment of bacteria able to use the natural analogs as growth substrates. Selection of such bacteria and elucidation of their biodegradation pathways could provide a remarkable source of novel enzymes for destruction or detection of synthetic analogs, including chemical agents.

The release of synthetic nitroaromatic compounds to the biosphere during the past century provided microbial populations with new sources of carbon, nitrogen, and electron acceptors, but new catabolic pathways had to evolve before the novel substrates could be exploited for growth. Pathways for degradation of novel compounds evolved through well-recognized processes of gene transfer, genetic mutation, and recombination and transposition (36) but the origins of the genes are mostly unknown. In some instances the pathways are poorly regulated and not very efficient, as if they have been assembled only recently. In some instances the bacteria that degrade explosives have only evolved the ability to use part of the molecule (71). It seems likely that some if not all of the pathways were assembled in soil bacteria by recruitment of portions of existing pathways for degradation of natural nitro compounds, but nothing is known about the possible links.

The purpose of this project was to explore the potentially much greater metabolic diversity of catabolic pathways for natural nitro compounds and AChE inhibitors. Our hypothesis was that the biochemistry is ancient, varied, widespread, and underappreciated. The metabolic diversity for degradation/destruction of natural compounds has very likely been the starting point for the recent and rapid evolution of pathways for degradation of synthetic chemicals.

A growing number of naturally occurring nitro compounds have been identified, but none consistently occur in high concentrations in the environment. Paradoxically, low detectable concentrations do not preclude the possibility of high flux in natural ecosystems. Recent discoveries suggest that, in some instances, the naturally occurring nitro compounds might have significant flux in the environment and the presence of such compounds has led to their exploitation by bacteria as growth substrates. For example, 4-nitroanisole-degrading bacteria were isolated from a mixture of farm and forest soils (65) that had no apparent prior exposure to 4-nitroanisole from anthropogenic sources. 4-Nitroanisole is produced by the basidiomycete, *Lepista deimii* (14), and it seems likely that the pathway for 4-nitroanisole degradation evolved in response to the presence of naturally occurring 4-nitroanisole in the soils. 4-Nitroanisole does not seem to accumulate in the environment, yet existence of a well-developed pathway for its degradation suggests that there is a significant flux of the compound in the soil.

A similar situation exists for other naturally occurring nitro compounds including 3-nitrotyrosine (3NTyr) (48) and 3-nitropropionic acid (3NPA) (47). In addition, we have isolated a wide range of different bacteria able to grow on synthetic nitroaromatic compounds from contaminated soils. We have not found such bacteria in nearby uncontaminated soils, which clearly indicates that the detection of the specific metabolic pathways is a strong predictor of the presence and flux of the chemical.

3NTyr is particularly interesting because it is the predominant product of the interaction of reactive nitrogen species with proteins and peptides (35). It has been found at the site of every inflammatory response in mammals (31), and it might also have a crucial role in cell signalling (23, 43). A SciFinder search for “nitrotyrosine” reveals 5930 references about its synthesis and physiological role, but nothing was known about its subsequent metabolism until our recent work (48). We focused our initial experiments on 3NTyr because of its wide distribution and key role in animals and because we have discovered soil bacteria that use it as the sole source of carbon, nitrogen and energy (48).

Degradation of 3NTyr is catalyzed by a pathway (Fig. 2) that is inducible and distinct from the tyrosine degradation pathways (48). The ubiquity of 3NTyr in biological systems and the

facile isolation of bacteria able to grow on it suggest that release of considerable quantities in the biosphere has led to the evolution and wide distribution of the pathway for degradation. Other nitro compounds in Fig. 1 can be readily detected when the organisms that produce them are studied in isolation, yet the compounds do not accumulate in the environment. It is highly likely that bacteria with appropriate catabolic pathways present in the same habitats account for the lack of accumulation. The overall objective of the research was to elucidate the mechanisms of metabolism of natural nitro compounds. We searched in a variety of ecosystems for bacteria that degrade different classes (nitroaromatic, nitroaliphatic, *N*-nitro, organophosphorus) of important natural compounds to determine whether the metabolism is based on recurring themes or tailored specifically to each molecule. Our purpose was to reveal the full range of metabolic potential for detection, synthesis and destruction of nitro compounds. The approach was subsequently applied to a natural organophosphate to determine whether the principles can be applied to other military unique chemicals.

Work performed

There were five specific goals of the research:

- Discovery of bacteria and determination of the biochemical mechanism(s) involved in the microbial metabolism of natural compounds beginning with 3NTyr followed by 3NPA.
    - Additional compounds were screened and added (5-nitroanthranilic acid (5NA), 1-nitro-2-phenylethane (NPE), chloramphenicol (Cam), *N*-nitroethylenediamine (NEDA) and cinnamoylphosphoramide (CPhos), etc.) or removed (pyrollnitrin, cyclopostin) from further study based on availability of the compounds or the results of preliminary enrichments.
- Determination of the molecular basis for the pathways by isolation of the genes and evaluation of their evolutionary origins
- Examination of the distribution of the catabolic pathways and determination of the extent to which the biochemistry is conserved across taxa
- Evaluation of the degree to which the ancient pathways for biodegradation of natural compounds provide the basis for rapid evolution of catabolic pathways for synthetic compounds of interest to DoD, including explosives, pesticides, and chemical agents
- Use of molecular information to design probes and primers to allow exploration of diverse habitats for the detection of the catabolic potential which will indicate the presence of synthetic pathways

Summary of the Most Important Results

At the start of our project we found literature reports of 210 natural nitro compounds (52) including analogs of nitrate ester, nitroaliphatic, nitroaromatic, nitramine and nitroheterocyclic explosives as well as several natural organophosphate compounds. In almost every case we attempted it was possible to isolate bacteria able to grow on the natural compounds, which means there are many enzymes evolved specifically for attack on the nitro and phospho groups.

We have established the biochemistry and molecular biology of the catabolic pathways for several of the molecules. The new enzymes and genes involved in the elimination of the key functional groups could not have been found by bioinformatics.

The story emerging from our findings with natural nitro compounds is that there is a vast and untapped metabolic diversity among the bacteria involved in synthesis and degradation of the millions of natural organic compounds. Such diversity can provide the basis for evolution of pathways for degradation or biosynthesis of new chemicals of interest to the DoD. The novel enzymes and genes also suggest an explanation for the existence of the daunting number of unknown and misannotated genes that make up over half the entries in the public databases (41, 55, 61, 66).

Many of the original tasks outlined in the proposal are complete while others have gone more slowly than expected. On the other hand, we have been able to pursue some unexpected opportunities such as the discovery of the first bacteria able to grow on nitroglycerin. Recently we isolated bacteria able to grow on DNAN, an insensitive explosive being phased in as a drop-in replacement for TNT.

Initial efforts were focused on extending our previous work with 3NTyr- and 3NPA-degrading bacteria as model systems to answer the above questions. We selected several other naturally occurring compounds to include in initial screens for bacteria that can grow on such compounds. The selection, based on similarity of the compounds to synthetic explosives and organophosphates, included NPE, a volatile phenyl-substituted nitroaliphatic compound produced by a variety of plants; 5NAA, a polar nitroaromatic compound produced by a variety of *Streptomyces*; Cam, a nitroaromatic antibiotic produced by streptomycetes; and NEDA, an *N*-nitro compound produced by *Streptomyces* that is an analog of the explosive ethylenedinitramine (Haleite or *N,N'*-dinitroethylene diamine). Additional compounds were included in later screens. Selective enrichments in which the above natural compounds were individually provided as the sole carbon, nitrogen and energy source were started with locally collected soils as inocula.

Following the initial discovery of new metabolic pathways the emphasis of the project shifted to understanding the diversity and distribution of the catabolic genes and their relatedness to genes involved in the degradation of synthetic nitro compounds. This purpose was to extend the understanding of biochemical and metabolic diversity involved in degradation of nitro compounds, and to reveal the potential for the genes to be recruited for evolution of bacteria able to degrade explosives. We also established a collaboration with a synthetic chemistry group to obtain Cphos, a newly discovered natural organophosphate. Declining costs of next-gen sequencing enabled us to take advantage whole genome sequencing to explore the context of catabolic genes in organisms isolated for this project. The details of the specific compounds and metabolic pathways follow.

3-Nitrotyrosine

The pathway for degradation of 3-nitrotyrosine (3NTyr) was described in *Burkholderia* sp. JS165 and *Variovorax paradoxus* JS171 (48). Although never found in high concentrations in the environment, 3NTyr is widespread in the biosphere and is used as a marker for inflammation. Bacterial degradation of 3NTyr is initiated by an α-ketoglutarate-dependent deamination to the presumed intermediate 4-hydroxy-3-nitrophenylpyruvate (HNPP). Decarboxylation of HNPP yields 4-hydroxy-3-nitrophenylacetate (HNPA). The key reaction is the denitration of HNPA to homoprotocatechuate which is degraded by well-characterized central metabolic pathways. Both

the initial deamination and the denitration of HNPA are specific for 3NTyr degradation. A gene (*denA*) that encodes an HNPA denitrase in *V. paradoxus* JS171 was cloned into *E. coli* and sequenced.

Differences in the biochemical characterization of HNPA denitrases from that of JS171, and the failure of *denA*-based primers to make a product from *Burkholderia* sp. JS165 suggested that *denA* represents only a portion of the genomic and biochemical variability involved in 3NTyr degradation even though all 3NTyr-degrading isolates tested used the degradation pathway found in the originally reported 3NTyr-degrading strains.

The PCR primers that amplified *denA* in JS171 amplified a similar product in a different *Variovorax* isolate, strain JS669. A *denA* deletion mutant was created in JS669 to confirm that the product of *denA* is involved in the denitration of HNPA. The mutant transforms 3NTyr more slowly than the wild-type, and has lost the ability to grow on HNPA (Fig. 3). The results indicate that *denA* is responsible for HNPA denitrase activity in the wildtype JS669.

The original degenerate *denA* primers identified *denA* genes in less than 25 percent of tested strains. New degenerate primers were designed to target conserved regions of *denA* in order to detect a broader range of 3NTyr-degrading bacteria. Tests of the new primers identified putative *denA* genes in some isolates that failed to yield products with the original primers. It appears that the new set of *denA* primers will facilitate detection of 3NTyr-degraders in environmental samples.

The amino acid sequences of DenA from *Variovorax* sp. JS669 and other bacteria with HNPA denitrase activity form a distinct group within the FAD monooxygenases (Fig. 4). Homologs from *Bradyrhizobium* and *Bordetella* when expressed in *E.coli* also catalyzed the denitration of HNPA. Thus HNPA denitrases are a group of novel enzymes which could be the source of the progenitor of enzymes that catalyze removal of nitro groups from synthetic nitroaromatic compounds and explosives. The work clearly establishes the function of a group of enzymes whose roles were previously unknown.

3-Nitropropionic acid

3-Nitropropionic acid (3NPA) is one of the most widespread of the naturally occurring nitroaliphatic compounds. As such, it served as a model compound for nitroaliphatic explosives such as FOX 7s in the early stages of this effort. It is structurally related to nitroethane, a nitroalkane manufacturing intermediate of mercury fulminate. It is a toxic component found in hundreds of legumes (27). Several enzymes have been described with catalytic activity towards 3NPA, but not in organisms that use 3NPA as a growth substrate. The most widespread of such enzymes are 2-nitropropane dioxygenases that were originally described in the fungus *Williopsis saturnus* variant *mrakii* (39) and have since been detected in many different organisms (over 2000 sequences were listed in GenBank in June, 2008) with the advent of whole genome sequencing. Despite the widespread occurrence, the physiological substrate was unknown at the start of this project.

3NPA-degrading bacteria were isolated from common garden soil (46). Analysis of partial 16S rRNA gene sequences identified two representative strains as *Cupriavidus* sp., designated JS190 and *Pseudomonas* sp., designated JS189. When strain JS190 was grown on 3NPA as the sole carbon, nitrogen and energy source, 70% of the initial nitrogen accumulated as nitrate and 2% accumulated as nitrite (Fig. 5).

The gene (*pnoA*) that encodes the key enzyme for removal of the nitro group was cloned and sequenced, and the enzyme was purified and partially characterized. Preliminary results indicated that the enzyme is a nitronate monooxygenase. BLAST analysis (3) of the amino acid sequence of PnoA identified the gene as a member of COG2070, a group of genes conserved across taxa and related to 2-nitropropane dioxygenase genes (Fig. 6) which were originally defined by flavoproteins purified from the yeasts *Neurospora crassa* and *Williopsis saturnus mrakii* for the ability to catalyze the transformation of 2-nitropropane. The early discovery resulted in application of the name 2-nitropropane dioxygenase to the family of enzymes, although the physiological substrate of the enzymes was never identified.

Many organisms contain multiple genes annotated as encoding 2-nitropropane dioxygenase. Despite the apparent widespread distribution of the COG, to date only proteins from 5 organisms have been purified and characterized to varying degrees. No evidence has been put forward that establishes 2-nitropropane as the physiological substrate of any of the enzymes included in COG2070. Very recently, the proposed a name change (19) from 2-nitropropane dioxygenase to nitronate monooxygenase with the new Enzyme Commission designation E.C.1.13.12.16., was approved based on recharacterization of purified proteins from the yeasts, *Williopsis saturnus* variant *mrakii* and *Neurospora crassa*. PnoA is biochemically distinct from the well-characterized nitronate monooxygenases, with only ~ 20 - 25% amino acid identity to the biochemically characterized enzymes.

We cloned 3 different putative 2-nitropropane dioxygenases from *Burkholderia phytofirmans* PsJN and *Pseudomonas aeruginosa* PAO1 that were among the proteins most closely related to propionate nitronate monooxygenase in the BLAST results. Enzyme assays indicated that the encoded proteins are highly specialized for propionate 3-nitronate (Table 1). The specificity of the enzymes for the nitronate tautomer of 3NPA (Fig. 7), confirmed that P3N is the physiological substrate rather than 2-nitropropane.

Ha et al. crystallized a 2-nitropropane dioxygenase from *P. aeruginosa* PAO1 and analyzed and aligned the amino acid sequence with several closely related 2-nitropropane dioxygenases (21), but did not determine the physiological substrate of the enzyme. When the propionate-3-nitronate monooxygenases described here were added to the alignment, only 3 of the highly conserved residues interacting with FMN along with the His152 identified as the catalytic base were conserved (Fig. 8). The evidence suggests that propionate-3-nitronate monoxygenases form a separate cluster within COG2070 and that many of the proteins annotated as 2-nitropropane dioxygenases, now nitronate monooxygenases, are in fact specific propionate-3-nitronate monooxygenases. The argument is supported by the fact that 3NPA is likely to be much more widespread in nature than 2-nitropropane and other nitroalkanes. Many of the current annotations generated by genome sequencing projects are wrong (18) and the situation with nitronate monooxygenase seems to be another example of annotation based only on modest sequence similarity without functional information.

Several *pnoA*-containing strains were tested for the ability to grow on 3NPA. All strains containing *pnoA*, but not the *E. coli* control lacking the *pnoA* gene, grew on 3NPA as a nitrogen source but not always as a carbon source. The result suggests the lack of a complete degradation pathway or inability to regulate such a pathway in some strains. It is possible that in some bacteria the presence of propionate-3-nitronate monooxygenase might be a detoxification mechanism similar to the proposed function in fungi and plants. It is also a plausible mechanism of scavenging nitrogen. There may be a continuum of enzymes with functions from protection to

growth represented in the diversity of genes in COG2070. The enzymes are clearly the most prevalent strategy for destruction of nitroaliphatic compounds in nature. In collaboration with biochemists from Georgia State University, we have established that P3N is the actual physiological substrate of the original yeast enzymes (16). P3N monooxygenase seems to serve as a detoxification mechanism in yeast that likely increases fitness during interspecies competition.

The work described above enabled us to obtain funding from COMBREX, a collaborative NIH- funded effort to establish the function of important, but misannotated and unknown bacterial genes in the public databases. We are leveraging the funding from COMBREX to establish the function of 10 related "2-nitropropane dioxygenases" with our partners from Georgia State University. The approach will support conclusions about the diversity of the enzymes and their applicability to compounds of interest to DoD.

1-Nitro-2-phenylethane

1-Nitro-2-phenylethane (NPE) is a naturally occurring, aromatic compound with a nitroaliphatic side chain that is produced by a variety of plants. *Burkholderia* sp. JS670 grows on NPE and 2-nitro-1-phenylpropane (NPB), a synthetic compound with a longer sidechain, using the compounds as the sole carbon, but not as a sole nitrogen source. Preliminary results indicated that a dioxygenase was involved in NPE degradation. Simultaneous induction experiments indicate that *Burkholderia* JS670 has similar degradation pathways for NPE and biphenyl. The result suggested that NPE is degraded via a pathway similar to dioxygenase-mediated toluene and biphenyl degradation upper pathway and a hydrolase-mediated lower pathway (Fig. 9). Following ring-opening, the original side chain is released from the ring-cleavage product as 3NPA. The dienoate fragment is further metabolized as a carbon source.

Mutagenesis experiments and qRT-PCR confirmed the activity of the NPE dioxygenase and the upregulation of both the NPE dioxygenase (74-fold) and the NPE hydrolase genes (230-fold), demonstrating that the putative NPE dioxygenase and hydrolase are catabolically important and necessary for the biodegradation of NPE (Fig. 10). Deletion of the NPE dioxygenase gene abolished growth on NPE. The NPE dioxygenase and hydrolase are distantly related to biphenyl dioxygenases and hydrolases.

There are many natural analogs of NPE and NPB. We used auxanography (51) to determine whether *Burkholderia* sp. JS670 and the NPB/NPE dioxygenase mutant can degrade various natural and synthetic analogs of NPE and NPB (Table 2). The wild type organism grows on many aromatic compounds 2 or more carbons in the sidechain while the mutant seems to be limited to 1 carbon sidechains. The results seem to reveal a common strategy not only for elimination of side chains containing nitro groups, but also other structures that would be bottlenecks to catabolic pathways.

Diphenylamine

Diphenylamine (DPA) is a naturally occurring phytocompound that is widely used as a stabilizer for explosives and as a precursor of dyes, pesticides, pharmaceuticals, and photographic chemicals. DPA is also formed as a byproduct during the manufacture of aniline. DPA and its nitrated derivatives are common contaminants at munitions-contaminated sites as well as at manufacturing sites. DPA is biodegraded under both aerobic and anoxic conditions but the mechanisms and the organisms responsible for the biodegradation were unknown.

Burkholderia sp. strain JS667 and *Ralstonia* sp. strain JS668 were isolated by selective enrichment from sediment from a DPA-contaminated stream (68). The isolates grew aerobically with DPA as the sole carbon, nitrogen, and energy source. During induction on DPA, stoichiometric amounts of aniline accumulated and then disappeared, which suggested that aniline is on the DPA degradation pathway. A 40 kb fragment containing the genes that encode the enzymes that catalyze the initial steps in DPA degradation was cloned from the genomic DNA of strain JS667. The *E. coli* clone catalyzed stoichiometric transformation of DPA to aniline and catechol. Transposon mutagenesis and the amino acid sequence similarity of putative open reading frames to those of well characterized dioxygenases support the conclusion that the initial reaction in DPA degradation is catalyzed by a multi-component ring-hydroxylating dioxygenase.

Genes that encode the complete aniline degradation pathway were found 12 kb downstream of the genes that encode the initial DPA dioxygenase. Reverse transcriptional PCR analysis confirmed the expression of each of the relevant dioxygenases in DPA grown cells of JS667. The results indicate that DPA is converted to aniline and catechol via angular dioxygenation and spontaneous rearomatization. Aniline and catechol are further biodegraded by the well established aniline degradation pathway. Genes that encode the complete DPA degradation pathway are on a 31 kb DNA segment. The sequence similarity and the gene organization suggest that the DPA degradation pathway evolved by recruitment of two gene clusters that encode DPA dioxygenase and an aniline degradation pathway (Fig. 11).

The organization of the genes that encode the DPA dioxygenase enzyme system is similar, but not identical to that of the well conserved *car* operon for degradation of carbazole, a natural compound structurally related to DPA. *carC* catalyzes the hydrolysis of the *meta*-cleavage products of 2'-aminobiphenyl-2,3-diol to anthranilate and 2-hydroxypenta-2,4-dienoate. The hydrolysis is not necessary in DPA degradation and *carC* is lacking in JS667. The presence of a truncated *carBb* in JS667, strongly suggests that the gene clusters involved in the initial steps in the DPA degradation pathway evolved by recruitment of the terminal dioxygenase gene from the carbazole degradation pathway (Fig. 12).

Recruitment of genes encoding DPA dioxygenase would be sufficient to allow common aniline degrading bacteria to grow with DPA as a sole source of carbon, nitrogen, and energy. The hypothesis is supported by several lines of evidence: the differences in G plus C content between the gene clusters that encode DPA dioxygenase and the aniline degradation components, several transposon remnants between the gene clusters, and that the aniline degradation operon is highly organized without superfluous genetic material relative to the DPA dioxygenase operon (Fig 11). The immature organization of the DPA operon seems to contradict the expectation that the genes encoding the degradation pathways of naturally occurring compounds are linked in operons without superfluous genetic material. It seems reasonable to speculate that the system was assembled at the field site in response to contamination by DPA. The genes of the assembled pathway appear to have been recruited from carbazole and aniline degradation pathways. Such assembly of degradative pathways in response to contamination has been observed in a chlorobenzene-degrading organism (44).

Bacteria able to grow on aniline, DPA, or carbazole as sole carbon, nitrogen, and energy source were isolated by selective enrichment from the same soil that yielded the original DPA-degrading strains. All of the DPA-degrading bacteria were able to grow on DPA and aniline, but not on carbazole. Neither carbazole nor aniline-degrading bacteria could grow on DPA.

PCR amplification was carried out with the primers based on nucleotide sequences of *dpaAa* from JS667. The internal region (800 bp) of *dpaAa* was amplified in the all the DPA degraders, but not in the carbazole degraders. The nucleotide sequences of the amplicons from the DPA-degrading strains were identical. The presence of identical DPA dioxygenase genes among distantly related DPA-degrading bacteria is indicative of horizontal gene transfer of the initial dioxygenase gene.

Similarly, a highly conserved 371 bp region of the aniline dioxygenase gene was amplified from the DPA- and aniline-degrading bacteria, but not from the carbazole-degrading isolates. The results indicate that DPA and aniline degraders have aniline dioxygenase genes. When PCR primers based on the gene cluster that encodes aniline dioxygenase in JS667 were used to search for homologs, identical DNA bands were observed in DPA degraders and one of the aniline degraders. The results indicate that they share highly similar aniline dioxygenase genes. The results are consistent with the proposal that genes from carbazole and aniline biodegradation pathways are the source of the genes that compose the DPA degradation pathway.

5-Nitroanthranilic acid

5-Nitroanthranilic acid (5-NAA), a natural nitroaniline, is produced by *Streptomyces scabies*. 5NAA can also be the starting material for synthesis of various nitroaromatic compounds and dyes. The introduction of the amino group into nitroaromatic compounds can make the resulting compounds more thermally stable. In the following series of aminonitrobenzene explosives, the thermodynamic stability increases from mono-amino-2,4,6-trinitrobenzene (MATB), to 1,3-diamino-2,4,6-trinitrobenzene (DATB), and 1,3,5-triamino-2,4,6-trinitrobenzene (TATB). Biodegradation pathways of nitro aromatic compounds and anilines are well documented, but nothing is known about that of nitroanilines.

Selective enrichment with 5NAA as the sole carbon and nitrogen source yielded *Bradyrhizobium* sp. JS329 (56). The first step in 5NAA degradation is deamination to form stoichiometric amounts of 5-nitrosalicylic acid (5NSA). Dialyzed and undialyzed cell extracts catalyze the initial reaction at almost the same rates. The result suggests that the deaminase requires no dialyzable cofactors such as flavin, metals, or electron donors. The further transformation of 5NSA doesn't require FAD, FMN, or extra electron donor, but does require Fe^{2+}. Transformation of 5NSA is inhibited by the flavin enzyme inhibitor diphenyliodonium, by the cytochrome P450 inhibitor metapyrone, and by chloramphenicol, and EDTA.

A genomic library of JS329 was screened for clones with the ability to transform 5NAA. Genes that encoded enzymes involved in 5NAA or 5NSA degradation were inactivated by insertion of the Tn5 transposon and the flanking regions were sequenced. The putative 5NAA deaminase is deeply branched and only distantly (34% amino acid identity) related to an uncharacterized M20/M25/M40 family peptidase from *Hyphomonas neptunium* ATCC 15444. 5NAA deaminase is the first hydrolase that removes an amino group from a benzene ring. When the gene that encodes 5NAA deaminase was cloned and over-expressed, enzyme assays revealed that the deaminase is specific for 5NAA. It did not attack anthranilate, 3-nitrobenzoate, 4-nitroaniline, 5-hydroxyanthranilic acid, or 4-nitroanthranilic acid, which indicates that the $-NO_2$ and -COOH substituents are essential for the deamination activity.

5NSA 1,2-dioxygenase is distantly related to gentisate 1,2-dioxygenase from *Oligotropha carboxidovorans* OM5 (29% amino acid identity) and salicylate 1,2-dioxygenase from *Pseudaminobacter salicylatoxidans* (22% amino acid identity). The enzymes share a conserved

histidine pair serving to anchor Fe^{2+} and a conserved domain. 5NSA dioxygenase is active against salicylate, 5-chlorosalicylate, and 5-bromosalicylate; and inhibited by iron chelators. The properties are similar to those of salicylate 1,2-dioxygenase (26), but salicylate 1,2-dioxygenase does not attack 5NSA (26). It is clear that 5NSA dioxygenase is specialized for the nitro-substituted compound.

Fumarate and pyruvate were products of enzyme assays that consumed 5NSA without additional cofactors. Two alternatives can account for the observed conversion: formation of a lactone accompanied by the removal of the nitro group, followed by hydrolytic ring cleavage to produce maleylpyruvate; or formation of a lactone without the release of nitrite, which is then hydrolyzed to maleylpyruvate with the removal of nitro group.

To identify the gene involved in the removal of the nitro group from the nitroaliphatic compound, the 5NAA fosmid DNA was sequenced, and one large fragment was annotated (58). Potential enzymes involved in the biodegradation of 5NAA are highlighted (Fig. 13). The proposed pathway is consistent with one reported for 5-halosalicylate (26). This study provides the first example for the denitration mechanism for the ring fission products, and provides the first clear evidence for the biodegradation of amino-nitroaromatic compounds.

5NAA deaminase (NaaA, Fig. 14) is the only hydrolase known to remove an amino group attached to the benzene ring, To determine biochemical properties of the enzyme, the gene was cloned in *E. coli*, and the protein was overexpressed. NaaA was purified by His-tag affinity chromatography. A collaboration was established with a lab specializing in structural biology and low resolution X-ray analysis of NaaA was obtained, but efforts are continuing to optimize the conditions for crystallization so that high resolution X-rays may be obtained. Potential substrates will be predicted by docking an array of nitroaniline explosives to the enzyme structure. Understanding of the substrate binding pocket will allow design of mutant enzymes that can bind explosive analogs of 5NAA.

4-Nitroaniline

The presence of the hydrolytic deaminase in the degradation pathway for 5NAA raised the question of whether the same mechanism can be applied to degradation of synthetic nitroanilines. 4-Nitroaniline (4NA) is a widely used synthetic intermediate that was previously reported to be biodegraded by an unknown mechanism. 4NA is an intermediate in the synthesis of dyes, antioxidants, pharmaceuticals, corrosion inhibitors and is very harmful to aquatic organisms (20, 64). It is structurally similar to natural compounds such as nitroanthranilic acids and synthetic explosives like *N*-methyl-4-nitroaniline (MNA), 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) that consist of nitro and amine groups attached to an aromatic ring. The multiply substituted aromatic ring makes such molecules hard to biodegrade.

Several studies related to 4NA biodegradation have been published over the years (60, 63, 76), but the complete biodegradation pathway of the 4NA remains to be established. Our objective was to rigorously establish the degradation mechanism, to determine whether the mechanism can be applied to explosives, and to discover the key genes and enzymes of 4NA biodegradation. Determination of the initial attack on 4-nitroaniline will provide insight about the effect of the carboxyl and nitro groups on the strategy for deamination.

Rhodococcus sp. strain JS360, capable of using 4NA as sole carbon and nitrogen source, was isolated from 4NA-contaminated soil. Simultaneous induction assays were used to infer possible pathway intermediates. During growth on 4NA nitrite accumulation is stoichiometric whereas only half of the theoretical ammonia accumulates, indicating that the ammonia is consumed for growth (Fig. 15). Oxygen uptake was stimulated by 4-aminophenol and 4-aminocatechol, but not 4-nitrocatechol, suggesting that the nitro group is released before the amino group. The chlorinated analog of 4NA, 4-chloroaniline also stimulated oxygen uptake. The result suggests that the enzyme that attacks the nitro group might also attack chloro substituents. 4NA-grown cells provided with 4-chloroaniline released stoichiometric amounts of chloride, suggesting that the first enzyme in the pathway is capable of attack on both chloro and nitro groups. Flavoproteins of the TC-FDM family are well known to be active with both chloro and nitro-substituted substrates (53).

The genome of JS360 was sequenced with the Illumina HiSeq2000 platform after a screen of the clone library failed to detect the gene responsible for the activity. The genome was annotated and primers were designed based on possible flavoprotein monooxygenases encoded in the genome. Genes encoding enzymes that are closely related to flavoproteins or to monooxygenases were cloned into *E. coli*. Preliminary tests indicated that an enzyme closely related to pentachlorobenzene monooxygenase initiates 4NA biodegradation. Analysis of the products of the enzymatic reaction is underway. The remaining clones will be tested in the same way to determine further steps and enzymes in the catabolic pathway (Fig. 16).

2-Nitroimidazole

2,4-Dinitroimidazole, is a powerful insensitive high explosive. 3-Nitro-1,2,4-triazol-5-one (NTO) is a related insensitive explosive that is currently emerging as a replacement for TNT and RDX. 2-Nitroimidazole (2NI), also known as azomycin, is a natural analog of 2,4-dinitroimidazole and NTO that is produced by actinomycetes and pseudomonads. 2NI inhibits the growth of bacteria, protozoa and a variety of anaerobic bacteria. Elucidation of the biodegradation pathway of 2NI was pursued to gain insight into potential mechanisms for dinitroimidazole biodegradation and for enzymatic removal of nitro groups from heterocycles in general.

Mycobacterium sp. JS330 was enriched from soil with 2NI provided as the sole carbon and nitrogen and energy source. Cultures grown on 2NI released stoichiometric amounts of nitrite and imidazol-2-one was detected in the culture medium (Fig 17). *Mycobacterium* JS330 also grows on imidazol-2-one as the sole carbon, nitrogen and energy source, which suggests that degradation 2NI is initiated by denitration to form imidazol-2-one.

The distribution of 2NI-degrading bacteria was determined in soil from a wide range of habitats from around US. Degradation of 2NI, accompanied by the accumulation of nitrite, was observed in 11 out of 13 samples. Six isolates obtained from the enrichments grew on 2-nitroimidazole or imidazol-2-one as the carbon and nitrogen source with the release of nitrite from 2NI. The isolates were most closely related to *Mycobacterium brisbanense*, *Rhizobium* sp., *Wautersia* sp., and *Phyllobacterium myrsinacearum*. Widespread ability to degrade 2NI indicates a substantial flux of 2NI in natural ecosystems and suggests an important ecological role of 2NI.

A fosmid that catalyzed the transformation of 2NI to nitrite and imidazol-2-one was sequenced. The gene that encodes 2NI nitrohydrolase (*nnhA*) was identified (Fig. 18) and the enzyme was characterized. 2NI nitrohydrolase catalyzes denitration of benznidazole but not 3- or

4-nitro substituted imidazoles, suggesting that the enzyme is specific for 2-nitro functional groups. The gene that encodes 2NI nitrohydrolase confers resistance to 2NI to *E. coli*. The finding reveals a new drug resistance mechanism in soil bacteria prior to its appearance in pathogens (2, 11, 12).

NnhA was purified by His-tag affinity chromatography. No added cofactors are required for activity of the purified protein. Conditions for crystallization of the enzyme are currently being optimized to enable x-ray crystallography. As the reaction catalyzed by the enzyme has not been previously described, it has been submitted to the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology (Enzyme Commission) for classification.

N-*Nitroethylenediamine*

N-Nitroethylenediamine (NEDA), is a natural nitramine compound, produced by the mushroom *Agaricus silvaticus*. NEDA is an analog of the insensitive high explosive ethylenedinitramine (EDNA or haleite, Fig. 19). EDNA can be either a primary explosive in detonators, as a booster charge, or as a secondary explosive. The *N*-nitro group found in NEDA is also a key component of RDX, HMX and other nitramine explosives. Determination of the unknown NEDA degradation pathway will provide insight to enable understanding of the metabolism of a wide range of nitramine explosives.

NEDA-degrading bacteria were isolated from soil for their ability to grow on NEDA as the carbon and nitrogen source (Fig. 20). It is clear that the isolates eliminate the nitro group as nitrite by an unknown mechanism. The genome of one of the NEDA-degrading isolates was sequenced. Analysis of the sequence will reveal whether a cytochrome P450 enzyme, similar to the XplA enzyme for RDX degradation (67) is involved. If similar genes are discovered, they will be examined to determine whether they are the progenitors of XplA. The biodegradation pathway will be studied using both biochemical and molecular biological approaches. The enzymes involved will be screened for the ability to denitrate nitramine explosives.

8- Nitroguanine

8-Nitroguanine (8NG) is a naturally occurring marker of DNA nitration. Nitroguanine is an analog of the explosive, 2,4-dinitroimidazole. The production of 8NG in biological systems is well understood, but nothing is known about its biodegradation. Because the subsequent metabolism and fate of the molecule in humans is a mystery, elucidation of the microbial degradation pathway could provide insight relevant to the biochemistry and toxicology. We synthesized 8NG and set up selective enrichments for bacteria able to use it as a growth source. We obtained an *Pseudomonas putida* isolate from soil that degrades 8NG under aerobic conditions.

The isolate grows well on guanine, but 8NG is toxic and is only partially degraded with stoichiometric release of nitrite. Experiments designed to determine the effects of 8NG on the cells indicate that 8NG inhibits cell growth irreversibly but no toxic metabolites accumulate in the medium. Various resins were tested for the ability to reduce the 8NG concentration in solution. Resin IRA 743 efficiently reduced the 8NG concentration but did not relieve the inhibition. Guanine supports growth of the bacteria but xanthine oxidase, which catalyzes the first step in the guanine degradation pathway, did not attack 8NG. We are currently determining the initial step in the 8NG degradation pathway and the source of inhibition during growth on 8NG alone.

Chloramphenicol

Chloramphenicol, an antibiotic compound produced by *Streptomyces*, is one of the best known naturally occurring nitroaromatic compounds. Despite much research dedicated to mechanisms of chloramphenicol resistance, the mechanisms for complete degradation of the molecule remains a mystery. Lingens et al. proposed a catabolic pathway, based solely on isolation and identification of metabolites (42), that has not been substantiated by biochemical or genetic evidence and in light of recent discoveries about degradation of synthetic nitroaromatic compounds, the previously proposed pathways warranted reexamination. Catabolic pathways that have evolved in soil bacteria to detoxify or exploit natural antibiotics represent a source of metabolic diversity. Genes that evolved for biodegradation additionally serve as a reservoir of antibiotic resistance determinants that may be transferred to pathogenic bacteria (12, 13).

We isolated *Nocardia* JS674 from soil based on its ability to subsist on chloramphenicol as a sole carbon and nitrogen source. Biochemical evidence suggests that the lower pathway for chloramphenicol degradation proceeds through 4-nitrobenzoate, and involves a partially reductive pathway that generates a hydroxylaminobenzoate intermediate. Enzyme assays confirm activity of an inducible 4-nitrobenzoate reductase and 4-hydroxylaminobenzoate lyase that are active when JS674 is grown on chloramphenicol (Table 3). The pathway is not induced when cells are grown on glucose or pyruvate.

The genome of JS674 was sequenced on the Illumina HiSeq2000 platform. Genes involved in the lower pathway for chloramphenicol degradation, including a 4-nitrobenzoate reductase and 4-hydroxylaminobenzoate lyase, were identified. The predicted enzymes from JS674 show high sequence similarity to characterized 4-nitrobenzoate reductase and 4-hydroxylaminobenzoate lyase enzymes from *Ralstonia pickettii* YH105 (48% and 60% similarity, respectively) and from *Pseudomonas putida* TW3 (44% and 57%, respectively) (30, 75). The enzymes involved in chloramphenicol degradation may be evolutionary precursors to those previously elucidated for related xenobiotic compounds such as 4-nitrotoluene (22), an intermediate in the production of TNT

Nitroglycerin

Nitroglycerin (NG, GTN) is the best known of the many and varied nitrate ester explosives including ethylene glycol dinitrate (EGDN), diethylene glycol dinitrate (DEGN), pentaerythritol tetranitrate (PETN), and nitrocellulose (NC). Recently, natural nitrate esters have been discovered in marine and freshwater systems. The alcyopterosins (49), the first recognized natural nitrate esters, are produced by soft corals, and the alkyl nitrates, particularly methyl nitrate and ethyl nitrate, most likely have a microbial origin (9, 70). The discovery of naturally occurring nitrate esters raises the possibility that some enzymes have evolved specifically for the catalysis of nitrate esters, but nothing is known about biodegradation of the natural nitrate esters.

Until recently, no microbes have been isolated that can grow on nitrate ester explosives. Previous research has shown that flavoproteins from bacteria and yeast can partially denitrate PETN and NG. In the case of NG, the endproducts are various mixtures of mono- and dinitroglycerins (MNG). In collaboration with Joe Hughes at Georgia Tech we isolated *Arthrobacter* sp. JBH1 that grows on NG (Fig. 21, (33)). We identified the flavoprotein that catalyzes the specific conversion of NG to 1MNG without accumulation of the dead end metabolite 2MNG. We sequenced the genome of strain JBH1 and used transposon mutagenesis to establish the identity of the key enzyme responsible for the further degradation of 1MNG (32).

The enzyme is closely related to glycerol kinase and seems to phosphorylate MNG. The phosphorylation enables the subsequent utilization and complete degradation of MNG by an unknown mechanism (Fig. 22). The discovery of the key enzymes sets the stage for determining the biochemical properties and substrate ranges of the enzymes and how the catabolic pathway evolved. The NG degradation pathway seems to be in the early stages of evolution with enzymes that are not optimized and not regulated. The system is thus well suited for advancing our understanding about how such pathways evolve in response to the appearance of new synthetic chemicals by recruitment of genes from existing pathways.

2,4-Dinitroanisole

The DoD has recently begun the production of insensitive explosives including IMX-101, IMX-104 and PAX-21. The key component is 2,4-dinitroanisole (DNAN) a drop-in replacement for TNT. Although DNAN is not known to be a naturally occurring nitroaromatic compound, it is similar to 4-nitroanisole, a natural nitroaromatic compound produced by fungi and 2,4-dinitrophenol (24DNP) which is produced in the atmosphere by nitration of 2-nitrophenol. The catabolic pathways are known for both of the natural analogs and it seems likely that the biodegradation pathway for DNAN evolved by recruitment and modification of the genes that encode degradation of the natural analogs that have been in the biosphere for a very long time.

We have recently isolated bacteria from Holston AAP that have the ability to grow on both DNAN and 24DNP, apparently by similar mechanisms. When the isolates are grown on DNAN 24DNP transiently accumulates in the medium (Fig. 23). Subsequent degradation of 24DNP leads to the release of the nitro groups as nitrite. The results are the first indication that DNAN can be biodegraded completely and they strongly suggest that the pathway is an extension of the 24DNP degradation pathway. Previous studies reported the accumulation of a dead-end metabolite under anaerobic conditions (54). Our hypothesis is that recruitment of a gene encoding a demethylase is necessary and sufficient for extending the pathway for degradation of 24DNP to enable degradation of the new explosive. The discovery of naturally occurring bacteria that degrade DNAN will provide the basis for prediction of biodegradation of the new explosive in the environment.

(S)-*Cinnamoylphosphoramide*

Because we were unable to obtain sufficient quantities of the natural organophosphates originally proposed we worked with the newly discovered cinnamoylphosphoramide (CPhos), an organophosphate isolated from a tunicate-associated *Streptomyces* (59). When CPhos was provided as a sole carbon, phosphorus, and nitrogen source in enrichments from seawater, tunicates, tunicate-associated fauna, and sponges, CPhos disappeared from the enrichments with the transient appearance of more polar metabolites. LC-MS analysis identified the major metabolite with mass of 179 as *p*-hydroxycinnamic acid methyl ester (HCAME), indicative of a loss of $CH_3PO_2NH_2$ (phosphoramidic acid methyl ester, Fig. 24).

Authentic HCAME was rapidly degraded by the enrichments when either CPhos or methamidophos, an organophosphate insecticide, and close structural analog to phosphoramidic acid methyl ester, was provided as the phosphorus and nitrogen sources (Fig. 25). In the absence of methamidophos or CPhos, HCAME was not degraded. The results indicate that methamidophos can replace phosphoramidic acid methyl ester as the nitrogen and phosphorus source. Neither phosphoramidic acid methyl ester nor CPhos are required to induce the degradation of methamidophos. The slightly faster degradation of CPhos in the presence of

methamidophos might indicate that nitrogen is limiting when CPhos is the sole carbon, nitrogen, and phosphorus source.

Labrenzia sp. JS676 and *Erythrobacter* sp. JS677 were isolated from agar plates made with seawater containing Cphos as the sole growth substrate. Both isolates degrade HCAME (as sole carbon source) when provided with methamidophos (sole phosphorus and nitrogen source) or demeton-S-methyl (sole phosphorus source). The growth of the bacteria using methamidophos or demeton-S-methyl as phosphorus/nitrogen source indicates that the isolates possess enzymes that allows mineralization and subsequent assimilation of organophosphates (Fig 26). The isolates clearly contain a new hydrolase that has evolved to cleave the natural organophosphate. The ability to degrade methamidophos and demeton-S-methyl suggests that the OP hydrolase has a broad substrate specificity.

The genomes of both isolates were sequenced on the Illumina HiSeq2000 platform. Preliminary assembly and annotation revealed the presence of numerous alkylphosphonate (*phn*) genes in both isolates. No alkylphosphonate genes are present in the 2 public *Erythrobacter* genomes. Alkylphosphonate utilization is a secondary source of phosphorus for many bacteria that is suppressed in the presence of inorganic phosphate. There is indirect evidence that alkylphosphonate enzymes can attack ester-linked organophosphate nerve agents. The *phnCDEFGHIJKLMNOP* operon from *E. Coli* (7) consists of the membrane-bound C-P lyase (*phnGHIJKLM*), transporter proteins (*phnCDE*) and several accessory and regulatory proteins. *phnE* in *E. coli* K-12 strains requires a specific 8 base pair deletion to activate the gene and allow expression of the *phn* operon (34). When *phnE* with the specific deletion was cloned into *E. coli* JA221, the strain was able to use diisopropylfluorophosphate as a phosphorus source (15). Diisopropylfluorophosphatase is the formal name of OPAA, one of the two widely studied enzymes that attack nerve agents.

Conclusions

The overall objective of elucidating the mechanisms of metabolism of natural compounds related to DoD compounds of interest has been accomplished. In almost every case that we studied, we were able to isolate organisms that could grow on the naturally occurring compound. In the cases where the natural compound is widespread, such as for 3NTyr and 3NPA, isolates able to degrade the compounds are also widely distributed and readily obtained. Other isolates were only found in specialized environments when the natural product was reported from only a limited number of sources. Such was the case with 2-nitroimidazole and and 5-nitroanthranilic acid.

Novel biochemistry for the biodegradation of the compounds was identified in most instances which was in turn linked to previously unrealized gene clusters. The work with 3NPA led to solving the 50 year-old mystery of the true physiological substrate and purpose of 2-nitropropane dioxygenase. In the case of DPA, we were able to identify the genetic components that combined to form the DPA degradation operon. The results have borne out our initial hypothesis that there is a remarkable amount of untapped metabolic diversity for degradation of natural compounds. Such diversity is the ultimate driver of the biological component of natural attenuation, provides the basis for biosensors, and serves as an unlimited source of biocatalysts for synthesis and destruction of DoD materials.

Ongoing Work

Several aspects of the project are still ongoing. Crystallization of 5NAA deaminase and 2NI nitrohydrolase for X-ray analysis will enable detailed characterization of the enzymes. Studies on the 4NA degradation pathway are continuing with cloning of genes likely to be involved in the pathway and analysis of their products for activity. Similarly we are analyzing the products of genes cloned from chloramphenicol- and nitroglycerin-degrading isolates in order to clearly establish their roles in the biodegradation pathways.

Recommendations

Further research on DNAN, CPhos and NEDA are aspects of this project that are likely to yield valuable insights into the fate of compounds of interest to DoD and additional efforts for these three compounds is recommended. DNAN is now being used in production of insensitive explosives. Bacteria from Holston AAP have the ability to grow on both DNAN and 24DNP. When the isolates are grown on DNAN, 24DNP transiently accumulates in the medium. The results are the first indication that DNAN can be biodegraded completely and they strongly suggest that the pathway is an extension of the 24DNP degradation pathway. Our hypothesis is that recruitment of a gene encoding a demethylase is necessary and sufficient for extending the pathway for degradation of 24DNP to enable degradation of the new explosive. The genome sequence of the isolate and determination of the degradation pathway will clearly establish our fundamental understanding about how a new catabolic pathway emerged by recruitment of genes from existing catabolic pathways for natural nitro compounds. In addition, the discovery of bacteria that degrade DNAN will provide the basis for prediction of biodegradation of the new explosive in the environment.

Two bacteria that grow on CPhos have been isolated from tunicates and we have clear evidence of cleavage of the phosphate linkage along with identification of the transient intermediate metabolites. The isolates also degrade methamidophos and demeton-S-methyl, two synthetic organophosphate insecticides with similar structures, which suggests that the OP hydrolase has a broad substrate specificity. The genome sequence has revealed the presence of alkylphosphonate genes that previous research has implicated in the destruction/detoxification of organophosphates. Overexpression of genes in *E. coli* to determine the enzyme structures, mechanisms and activities toward other organophosphates will help to evaluate their potential to inform design of more effective decon/demil biocatalysts.

In spite of substantial efforts by several labs, the evolutionary origins of the cytochrome P450 that catalyzes the key denitration of RDX by various bacteria is a compelling mystery. It is still impossible to predict the biodegradation of RDX at ranges because the distribution of the bacteria is unpredictable. Isolates able to grow on NEDA release ammonia and nitrite during the degradation, which indicates clearly that the process involves an enzymatic denitration analogous to the denitration of RDX. The draft genome sequence of a NEDA-degrading bacterium contains a number of cytochrome P450 genes that are candidates for the denitration of NEDA. Transposon mutagenesis can be used to identify the key genes in the NEDA degradation pathway. Expression in *E. coli* should allow the enzyme mechanisms and substrate ranges to be determined to provide insight about the biochemistry. Their phylogenetic relationship to the key genes in the RDX degradation pathway will provide insight about the evolutionary origins of the ability to degrade RDX. The advance in understanding will enable improved predictions of RDX behavior and bioremediation in the environment.

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Figures

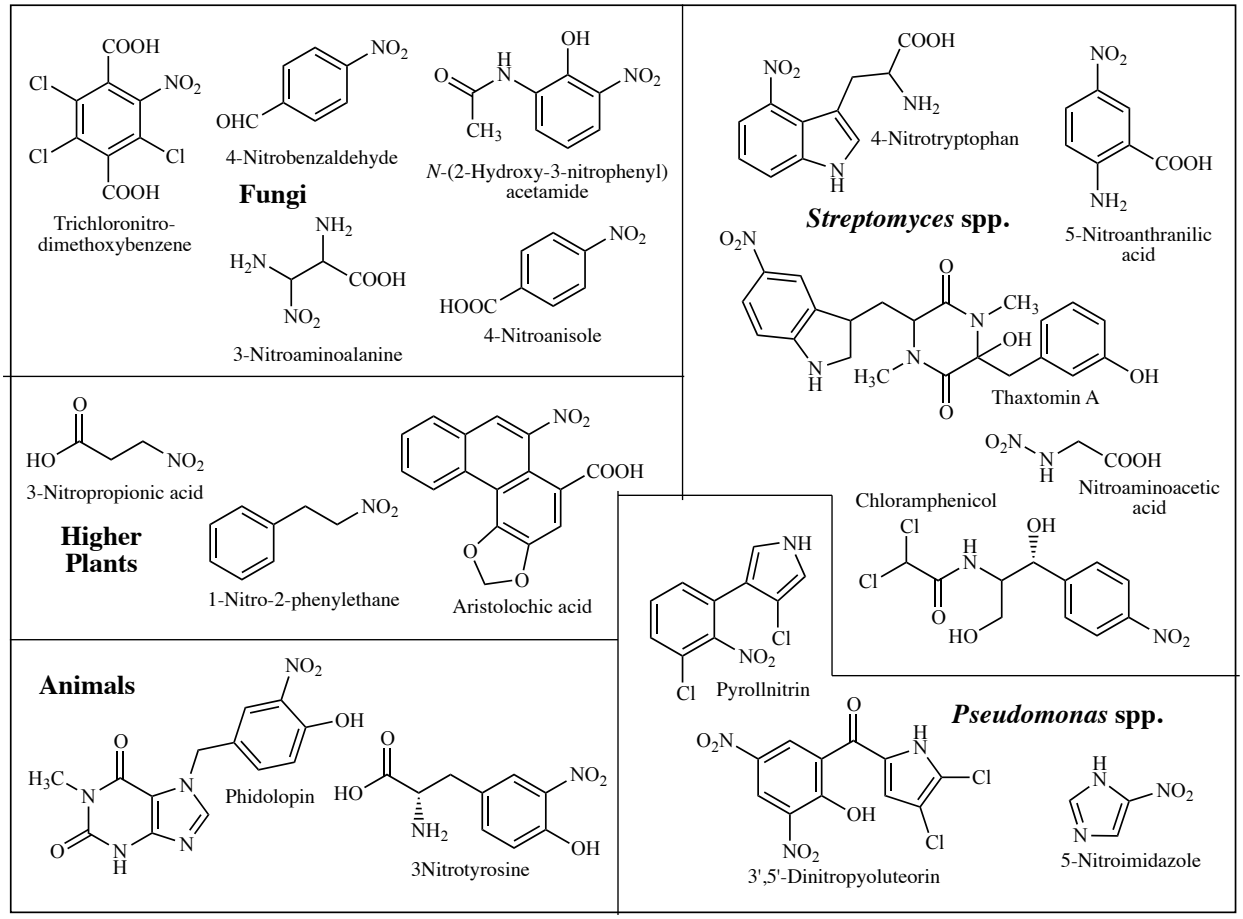


Figure 1. Representative examples of naturally occurring nitroaromatic and nitroaliphatic compounds. Over 220 named nitro compounds are found in the literature.

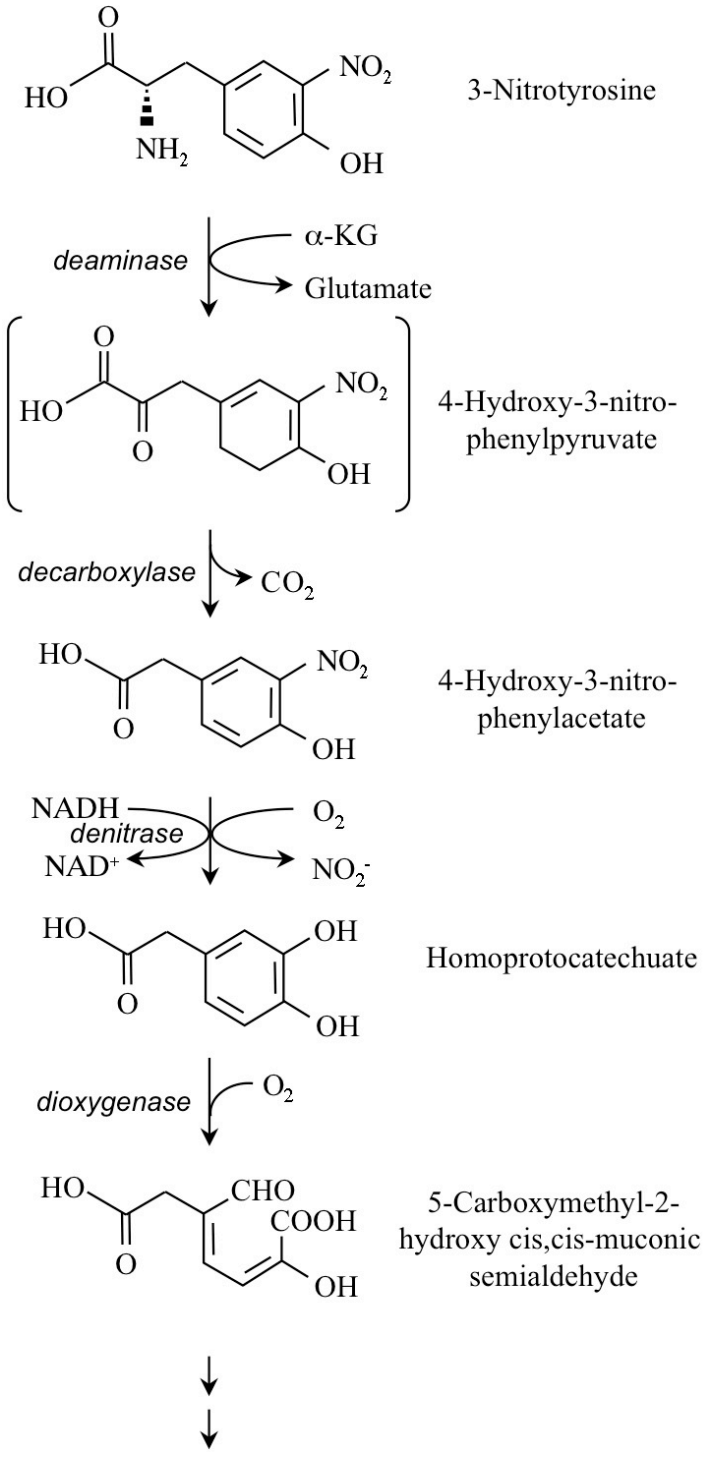


Figure 2. Catalytic pathway that supports growth of bacteria on 3-nitrotyrosine. Modified from (48).

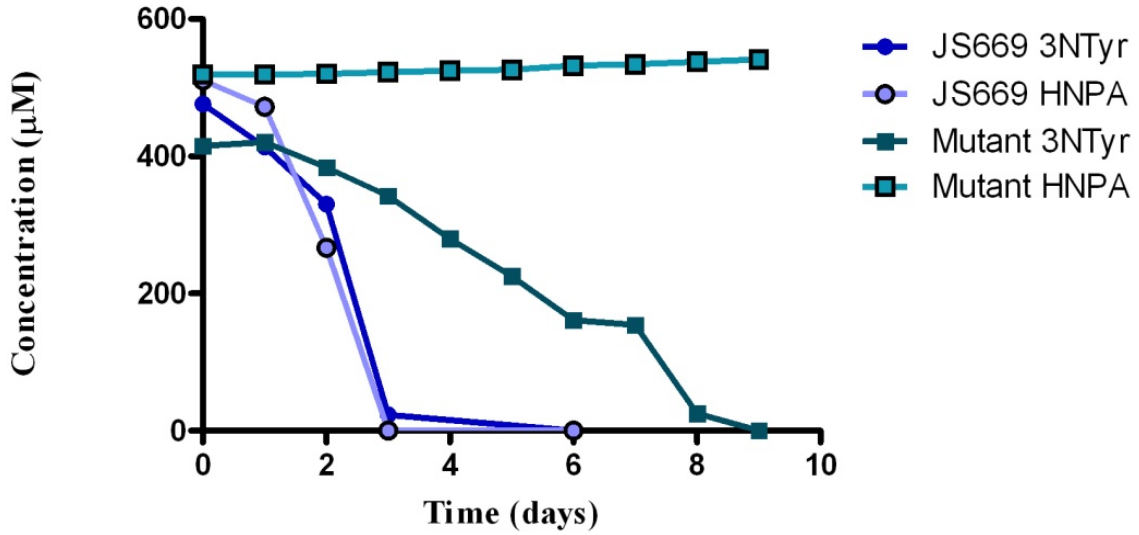


Figure 3. Transformation of 3NTyr and HNPA by *Variovorax* sp. JS669 wildtype and *denA* deletion mutant.

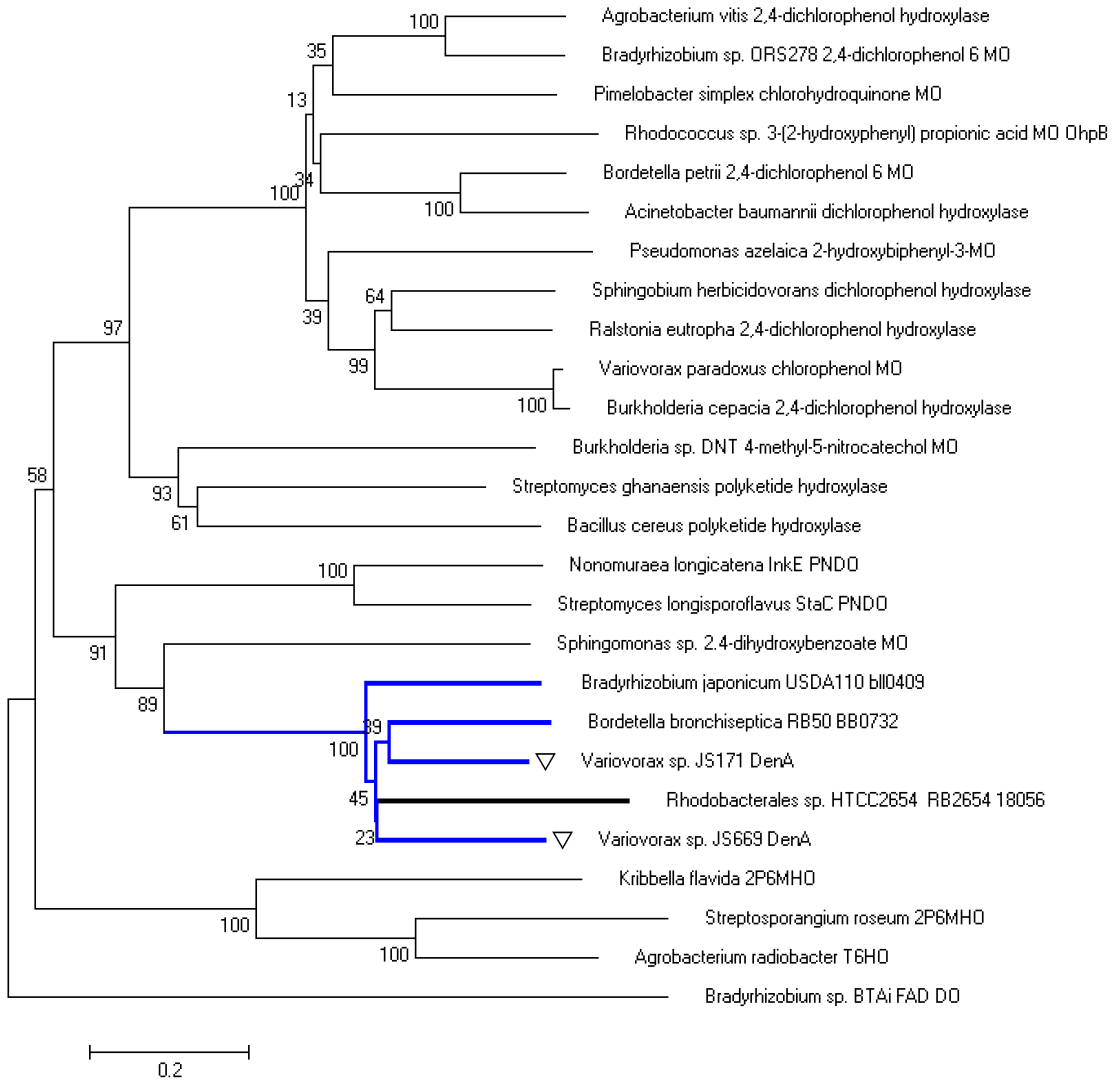


Figure 4. Phylogenetic analysis of DenA and nearest neighbors. Distance is in amino acid substitutions per site.

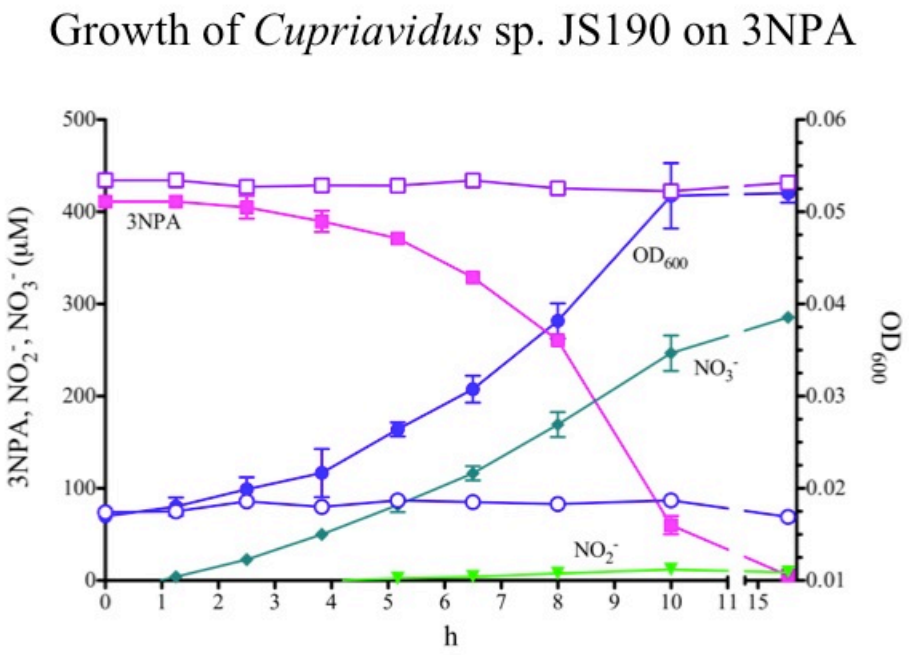


Figure 5. Cupriavidus sp. JS190 grows on 3NPA as a sole carbon, nitrogen, and energy source with the release nitrate and nitrite (46). Squares, 3NPA; circles, OD600; diamonds, nitrate; inverted triangles, nitrite; solid symbols, JS190; open symbols, uninoculated control.

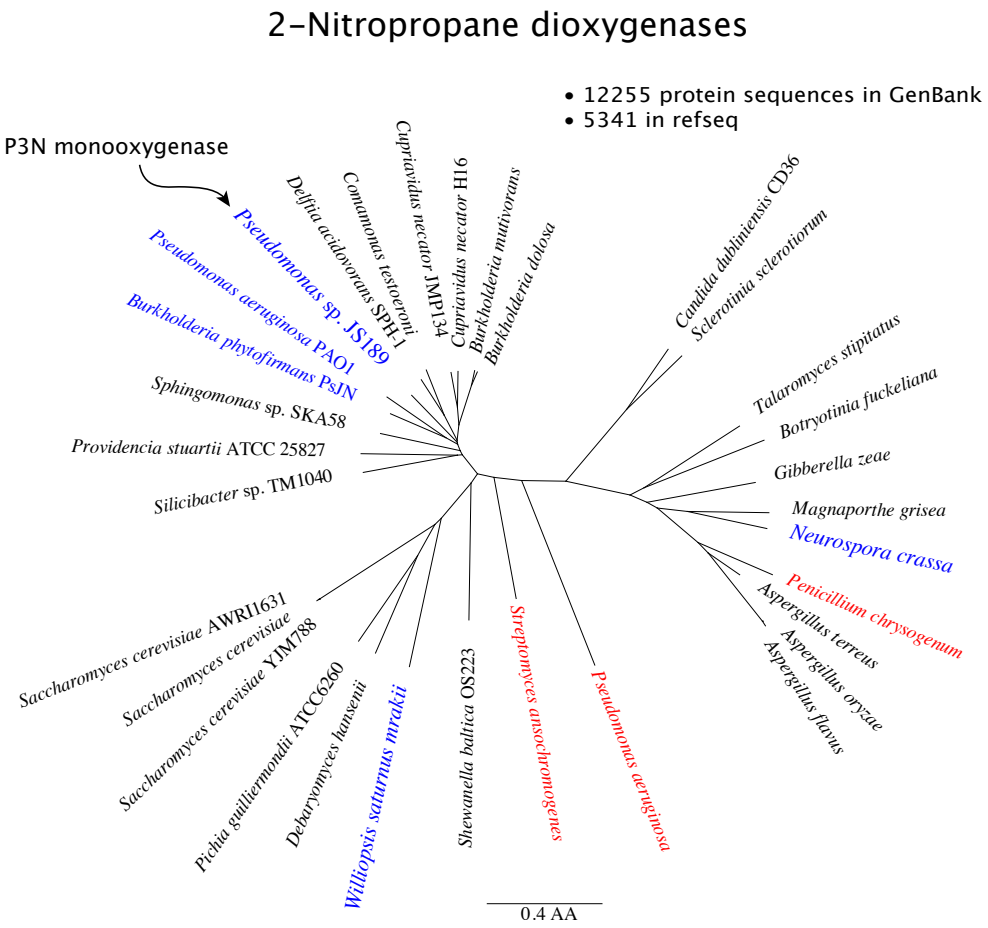


Figure 6. Tree of BLAST-P results for P3NO from the 3NPA-degrading *Pseudomonas* sp. JS189 with additional nitroalkane degradation proteins. Products of Bphyt_4212, Bphyt_6745, and PA4202, cluster with P3NO from JS189 (blue). Proteins from the yeasts *Neurospora crassa* and *Williopsis saturnus mrakii* (both in blue) are more distantly related, and were the sources for the first identified 2-nitropropane dioxygenases. Crystal structures have been obtained for 2-nitropropane dioxygenase from *P. aeruginosa* PAO1 (in red) and for nitroalkane oxidase from *Streptomyces ansochromogene*s (red). No sequences are available for P3NO from *Penicillium atrovenetum*, however *Penicillium chrysogenum* (red) is reported to be highly active against nitroalkanes and contains a gene annotated as 2-nitropropane dioxygenase.

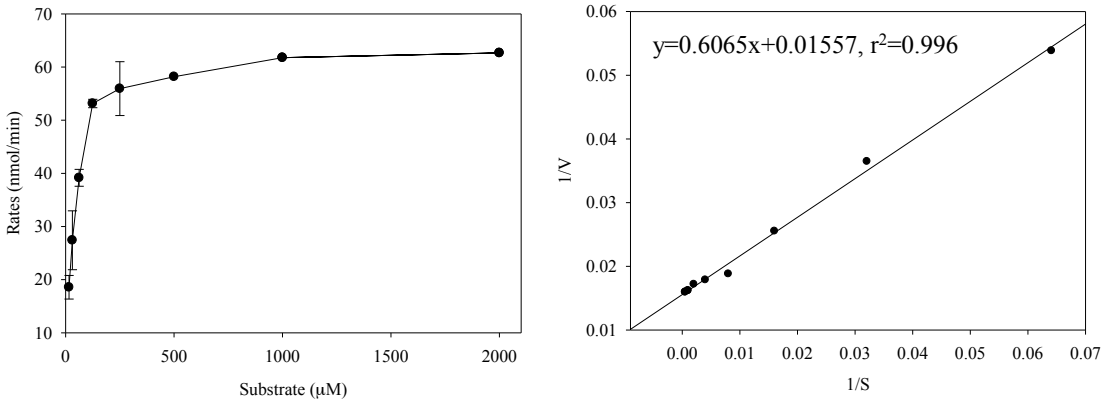


Figure 7. Michaelis-Menten kinetics of the enzyme encoded by PA4202 for propionate 3-nitronate and Lineweaver-Burk plot (Km = 39 uM, Vmax= 64 umol/min/mg protein).

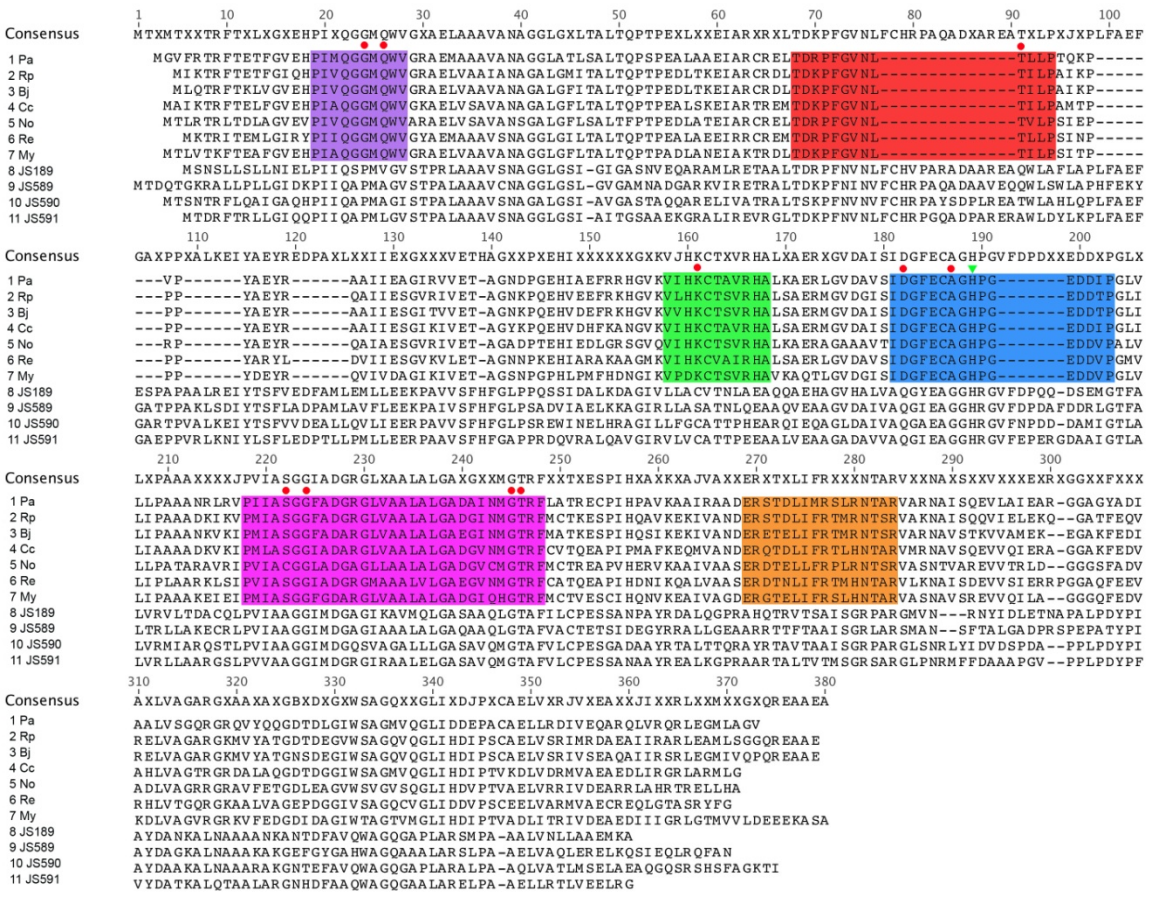


Fig. 8. Alignment of 2NPD and P3N oxidizing-enzyme (46). Sequences 1 – 7, motifs (colored blocks), highly conserved residues that interact with FMN (red circles), and proposed catalytic base (inverted green triangle) as in Ha et al. (21). Sequences 8 – 11 are propionate-3-nitronate monooxygenase from this work. Abbreviations (locus tag): Pa, *P. aeruginosa* (PA1024); Rp, *Rhodopseudomonas palustris* BisB5 (RPD_2021); Bj, *Bradyrhizobium japonica* USDA 110 (bll3143); Cc, *Caulobacter crescentus* CB15 (CC2990); No, *Nocardiodes* sp. JS614 (Noca_4138); Re, *Cupriavidus necator* JMP134 (Reut_B3609); My, *Mycobacterium* sp. P101 (Q5W8V2).

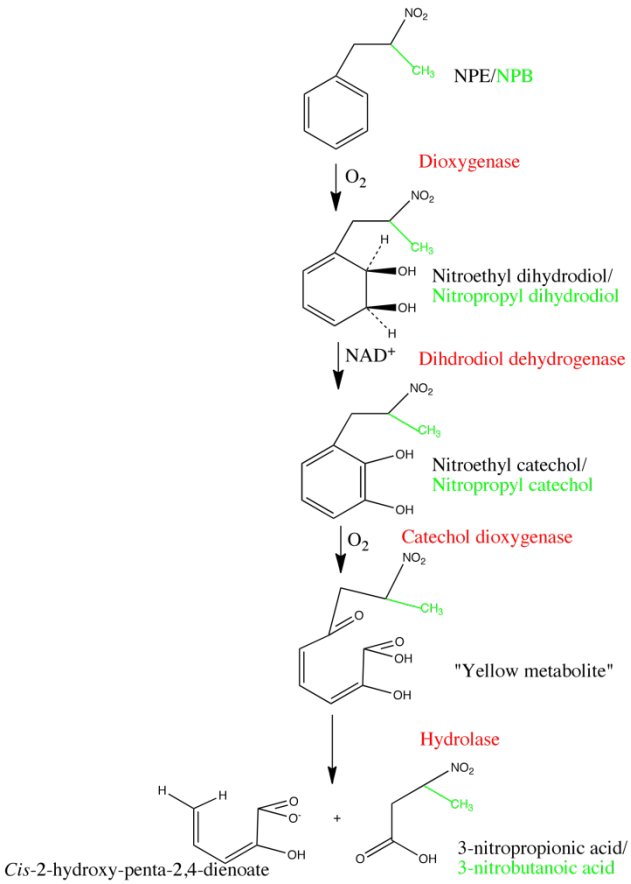


Figure 9. Proposed pathway for degradation of NPE (in black) and NPB (in green). Proposed enzymes are labelled in red.

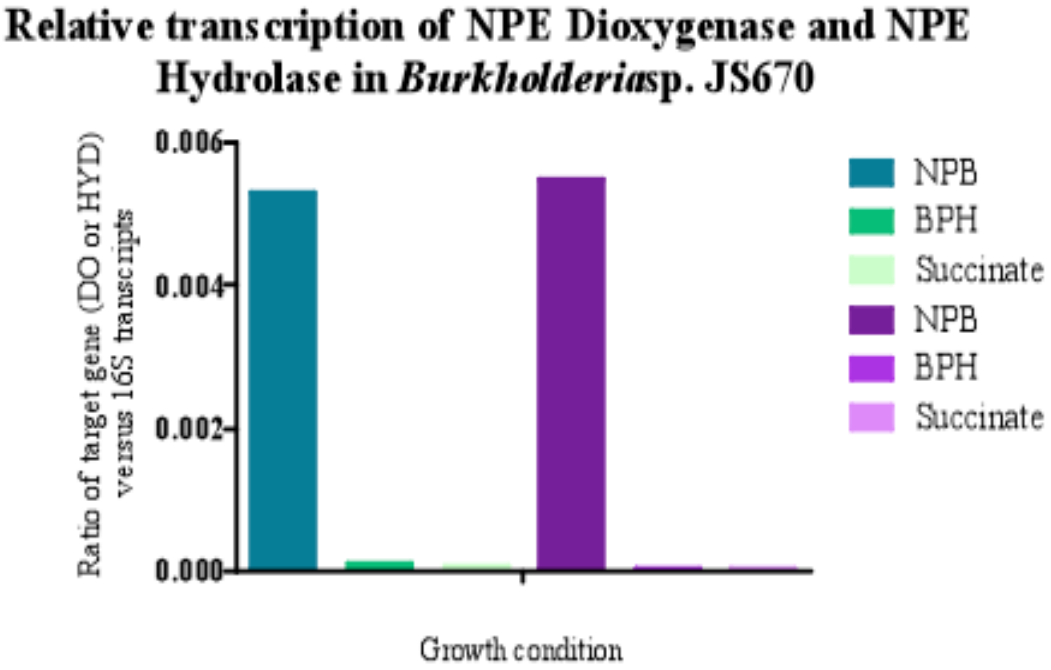


Figure 10. Relative transcription of NPE dioxygenase (green) and NPE hydrolase (purple) in NPB-, biphenyl- and succinate-grown cells.

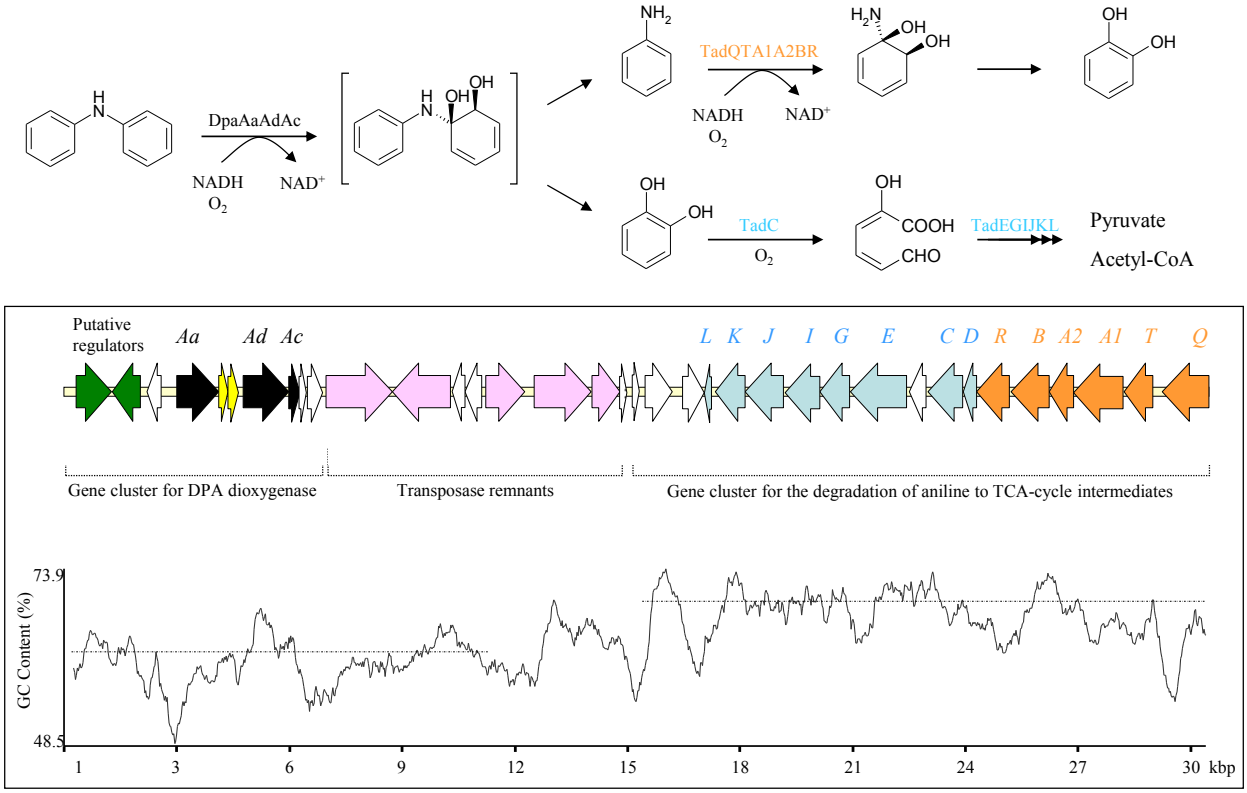


Figure 11. Biodegradation pathway of DPA (upper panel) and the genes (lower panel) involved in the degradation. The G+C content indicates that the genes for the DCA dioxygenase and the genes for aniline degradation came from two different sources. Modified from (68).

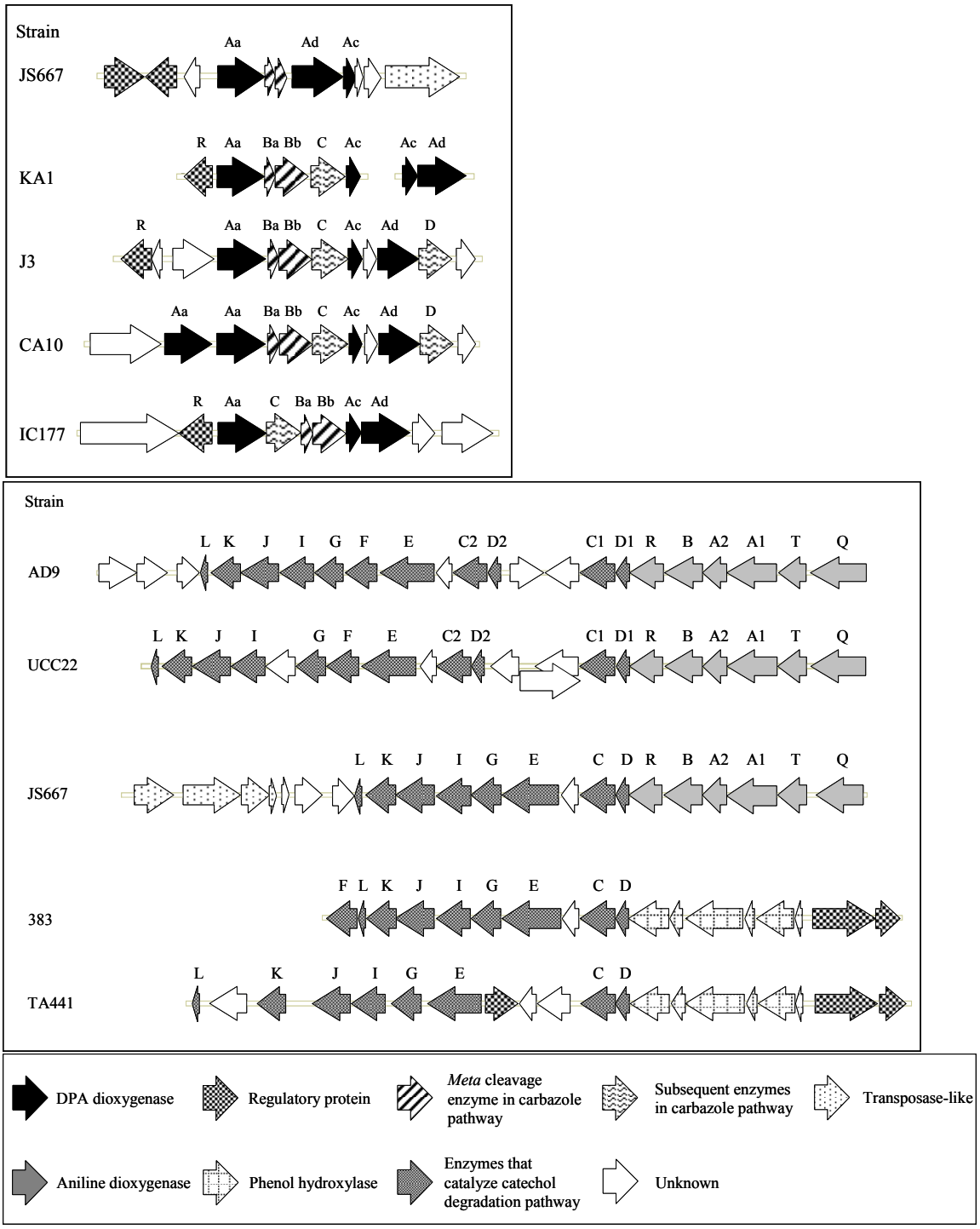


Figure 12. Top panel: organization of the genes that encode DPA dioxygenase from *Burkholderia* sp. JS667 and carbazole dioxygenases from *Novosphingobium aromaticivorans* IC177, *Sphingomonas* sp. KA1, *P. resinovorans* CA10, and *Janthinobacterium* sp. J3 Lower panel: organization of genes that encode the aniline degradation pathway from *Burkholderia* sp. JS667, *Delftia tsuruhatensis* AD9 and *P. putida* UCC22 and that encode the phenol degradation pathway from *Burkholderia* sp. 383 and *Comamonas testosteroni* TA441 (B). Modified from (68)

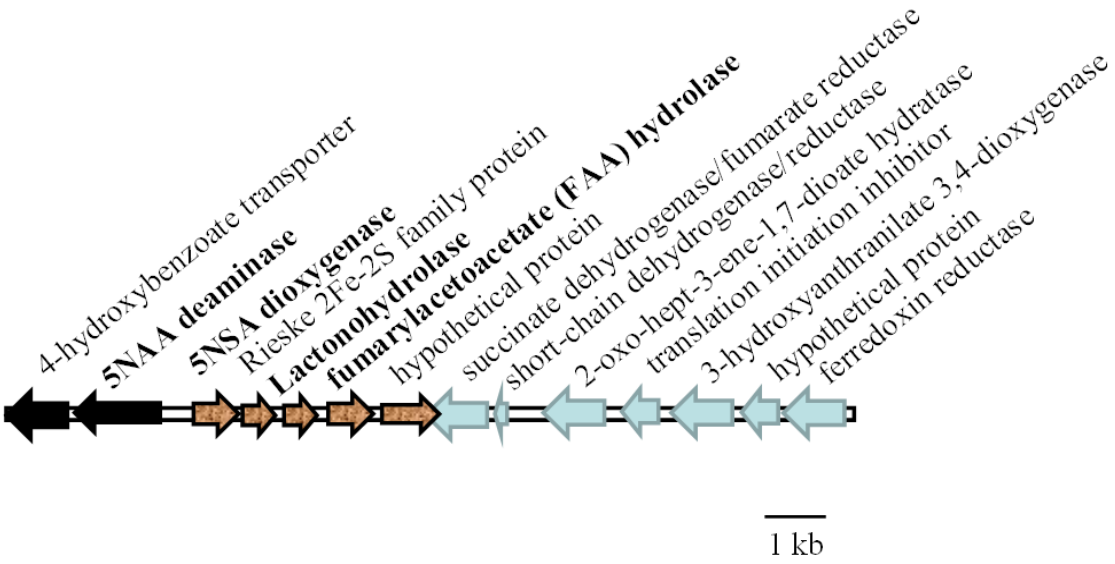


Figure 13. A fosmid that encodes the complete degradation of 5NAA was sequenced. Highlighted genes are involved in 5NAA degradation. Modified from (58).

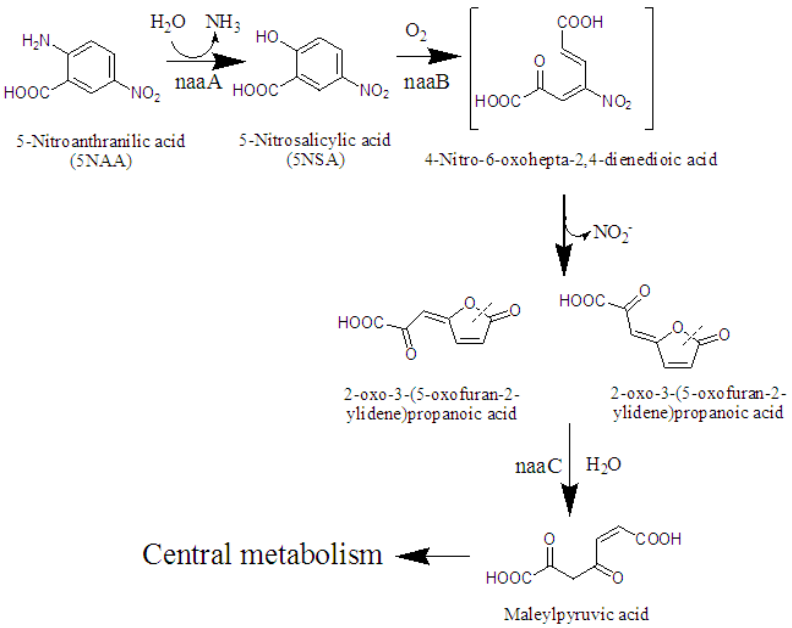


Figure 14. Degradation pathway for 5NAA. NaaA is the only hydrolase known to remove an amino group from a benzene ring. Modified from (58).

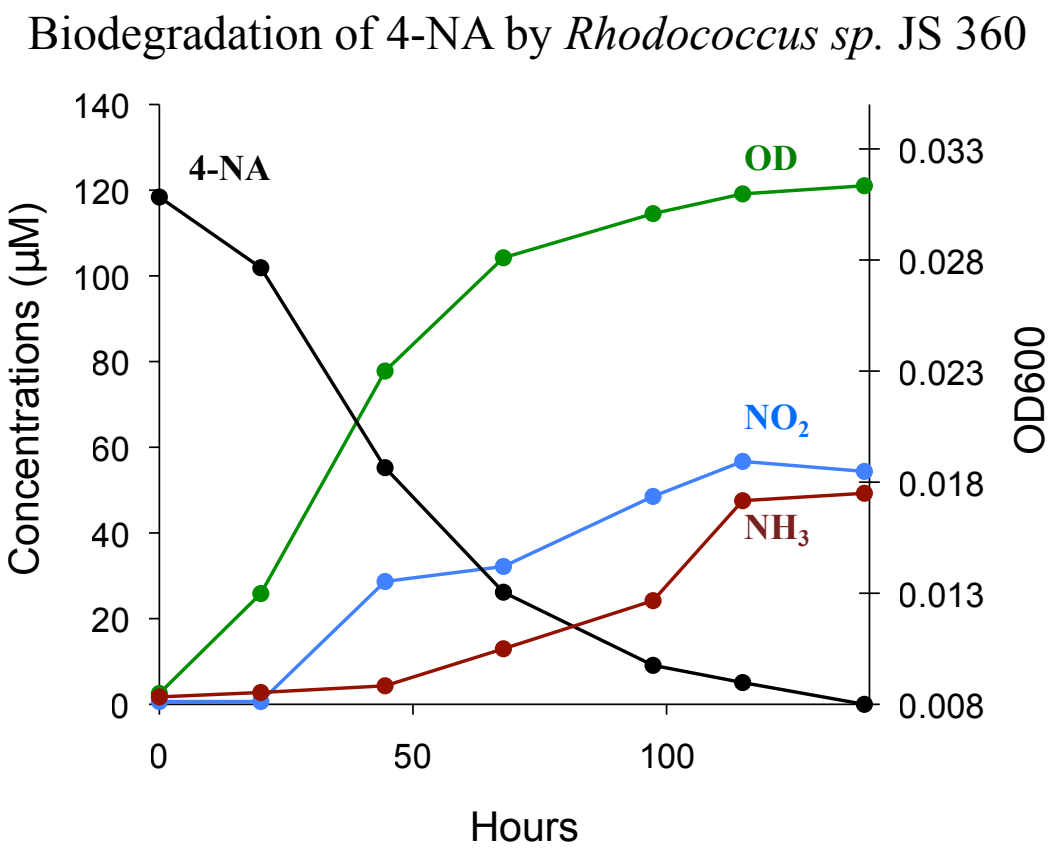


Figure 15. *Rhodococcus* sp. JS360 grows on 4NA with the release of nitrite and ammonia.

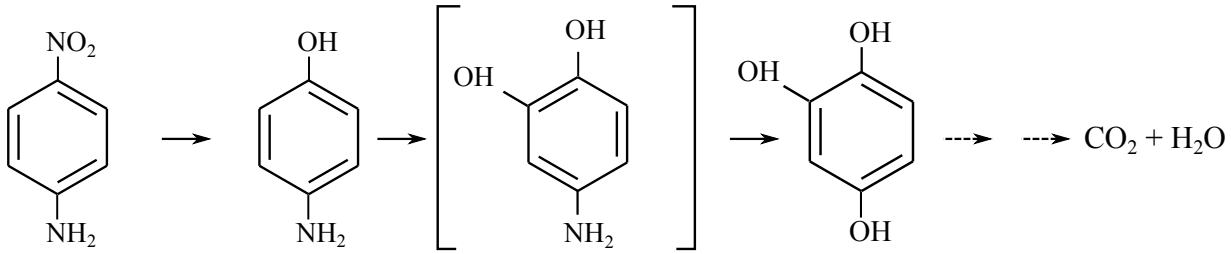


Figure 16. Proposed pathway for degradation of 4NA.

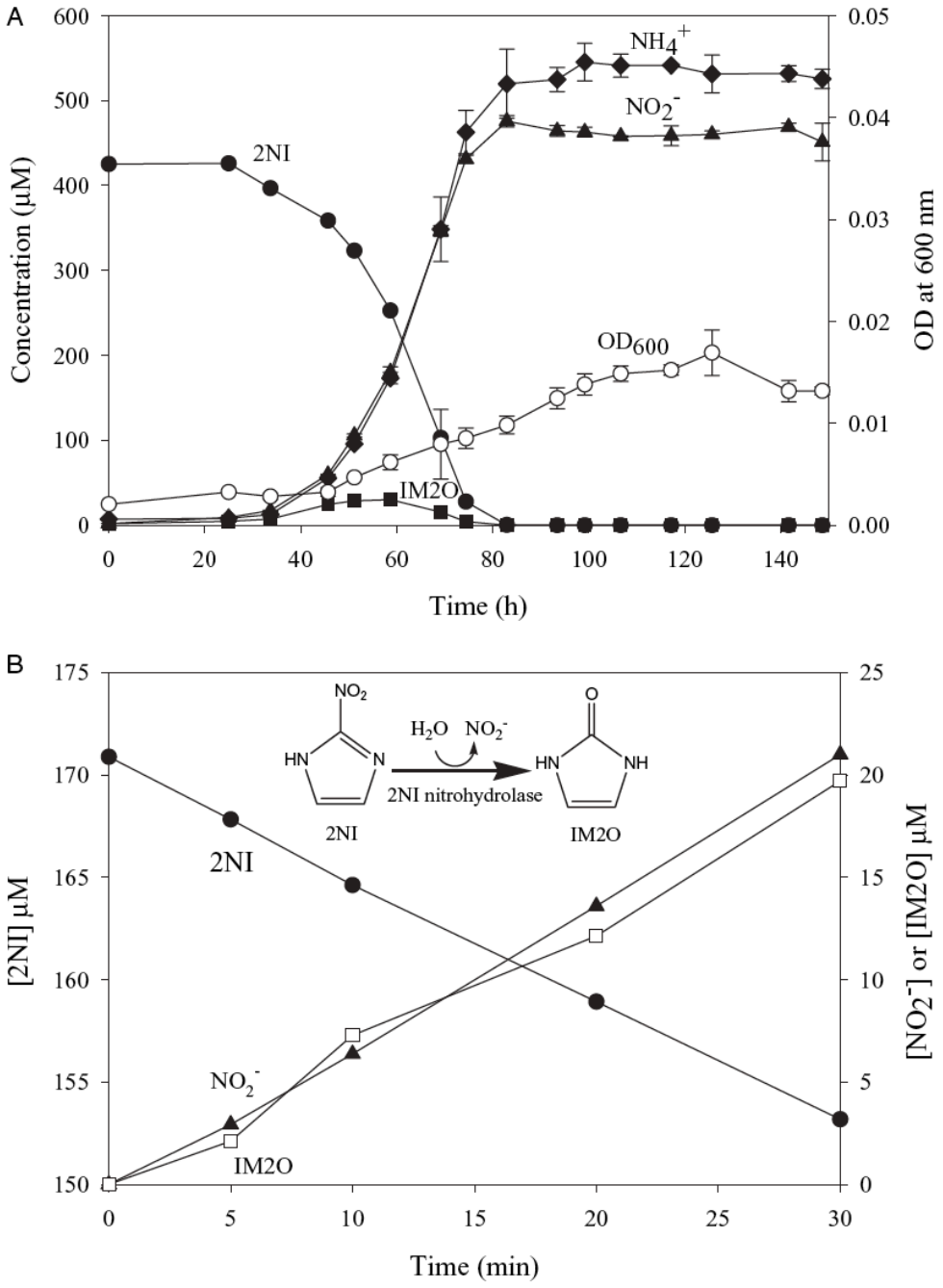


Figure 17. Initial loss of nitrite from 2-nitroimidazole results in the accumulation of imidazol-2-one (57). (A) Growth of Mycobacterium JS330 on 2NI as the sole carbon, nitrogen and energy source. ●, 2NI; ◆, NH4+; ○, OD600; ▲, NO2; ■, IM2O. (B) Transformation of 2NI by 2NI nitrohydrolase heterologously expressed in *E. coli*. ●, 2NI; ▲, NO2-; □, IM2O.

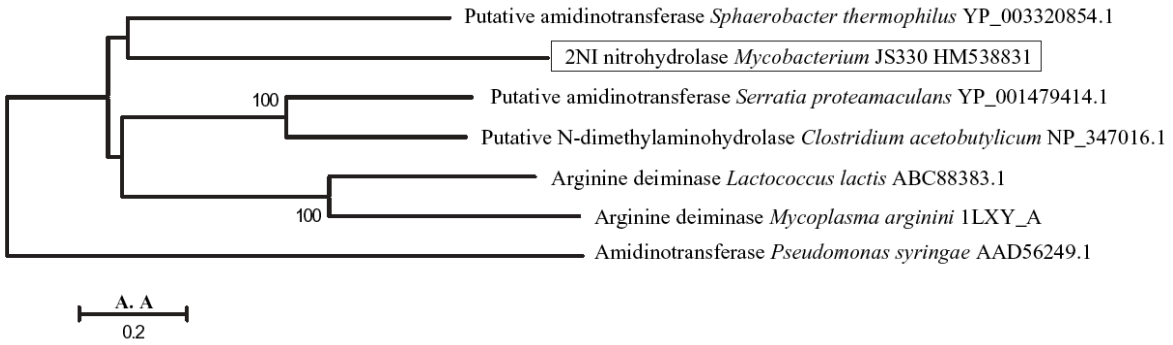
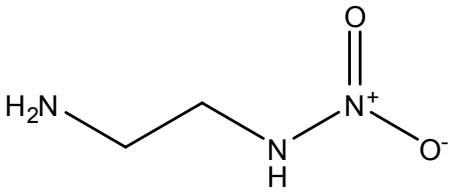


Figure 18. Phylogenetic tree for 2NI nitrohydrolase (57). Scale bar denotes 0.2 amino acid substitutions per site. GenBank accession numbers are indicated at the end of each sequence.

NO_2-NH_2-CH_2-CH_2-NH-NO_2

Ethylenedinitramine
(EDNA or Haleite)



N-nitroethylenediamine

Figure 19. *N*-Nitroethylenediamine is structurally related to the explosive ethylenedinitramine.

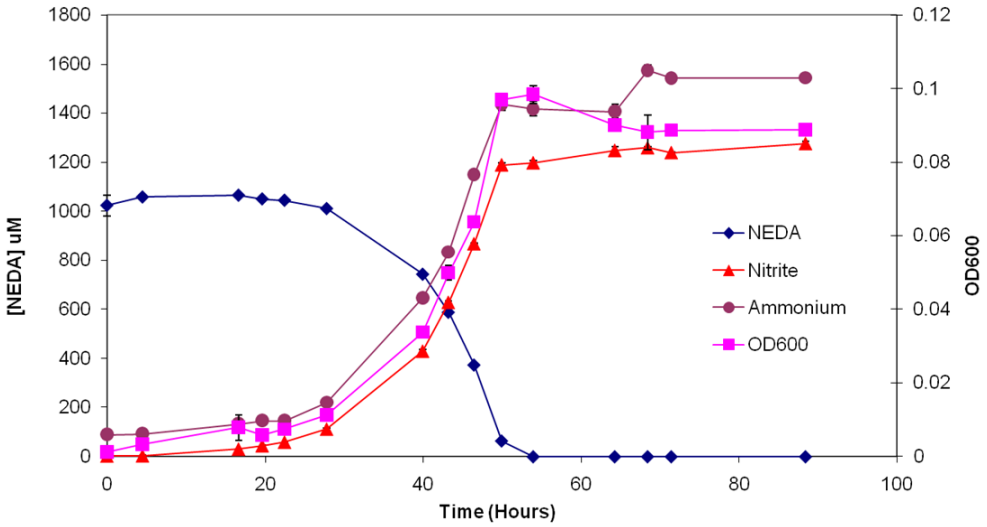


Figure 23. Bacteria are able to grow on NEDA with the release of both nitrite and ammonia.

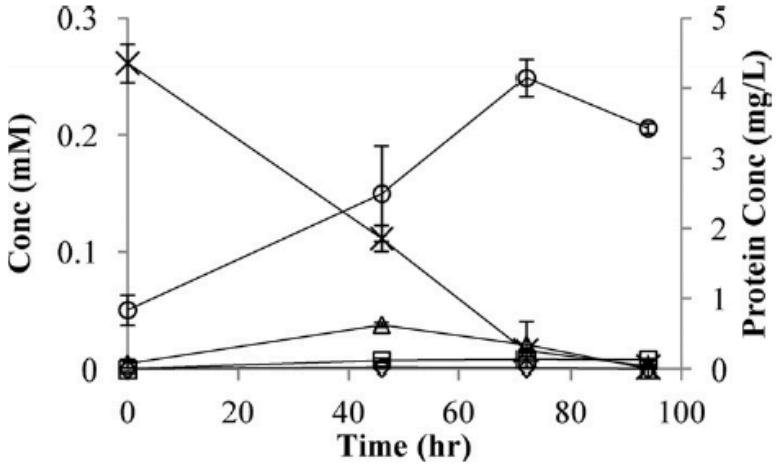


FIG. 1. Growth of strain JBH1 on NG. ×, NG; △, 1,2DNG; ◇, 1MNG; □, 2MNG; ○, protein.

Figure 21. *Arthrobacter* sp. JBH1 grows on nitroglycerin as a sole carbon, nitrogen, and energy source (33).

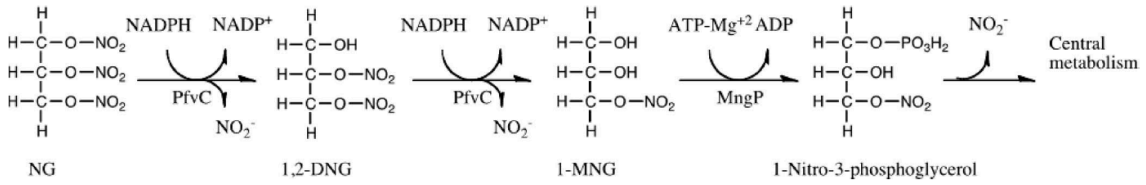
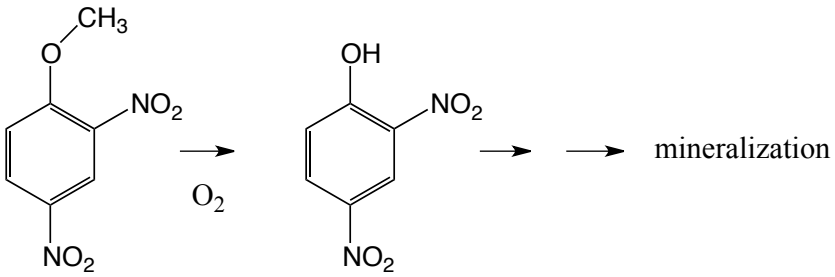


FIG 5 Proposed transformation of NG to 1-nitro-3-phosphoglycerol.

Figure 22. An enzyme related to old yellow enzyme (PfvC) catalyzes the initial denitration of nitroglycerin to 1-mononitroglycerol. Mononitroglycerol kinase (MngP) phorphorylates mononitroglycerol before release of the final nitro group (32).



DNAN 2,4-Dinitrophenol

Figure 23. Initial aerobic attack on DNAN leads to the transient accumulation of 2,4-dinitrophenol.

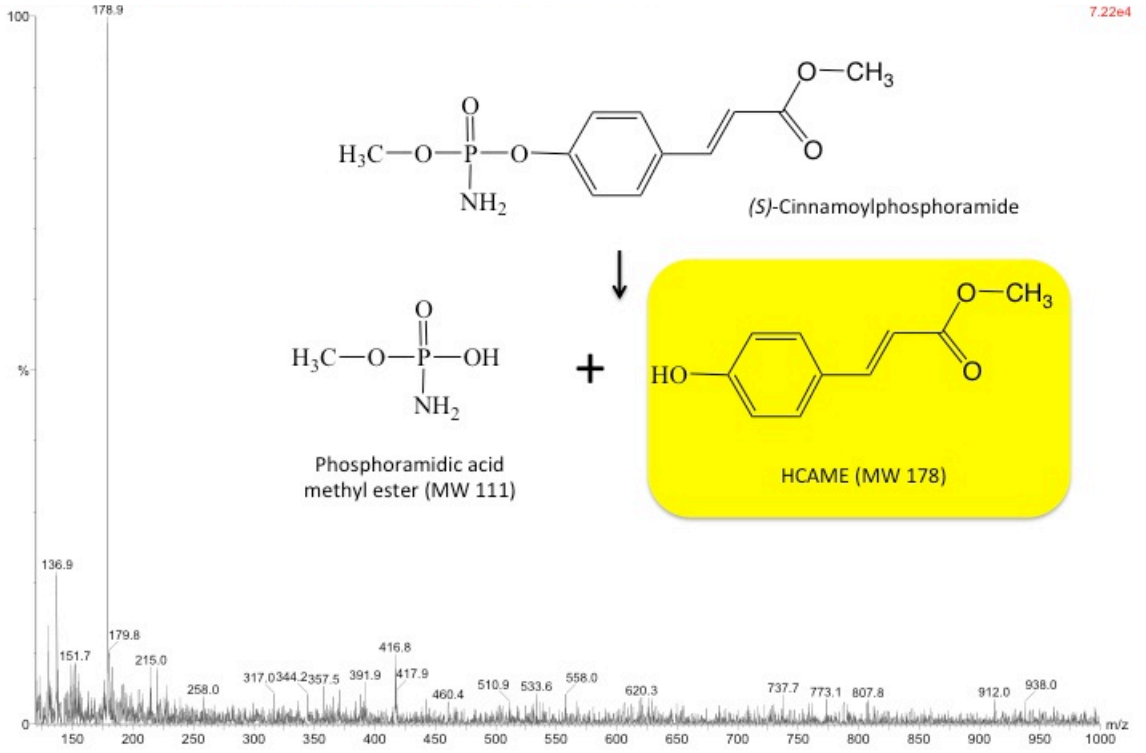


Figure 24. *p*-Hydroxycinnamic acid methyl ester (HCAME) accumulated transiently in cultures provided with CPhos as the sole carbon, nitrogen, phosphorus, and energy source.

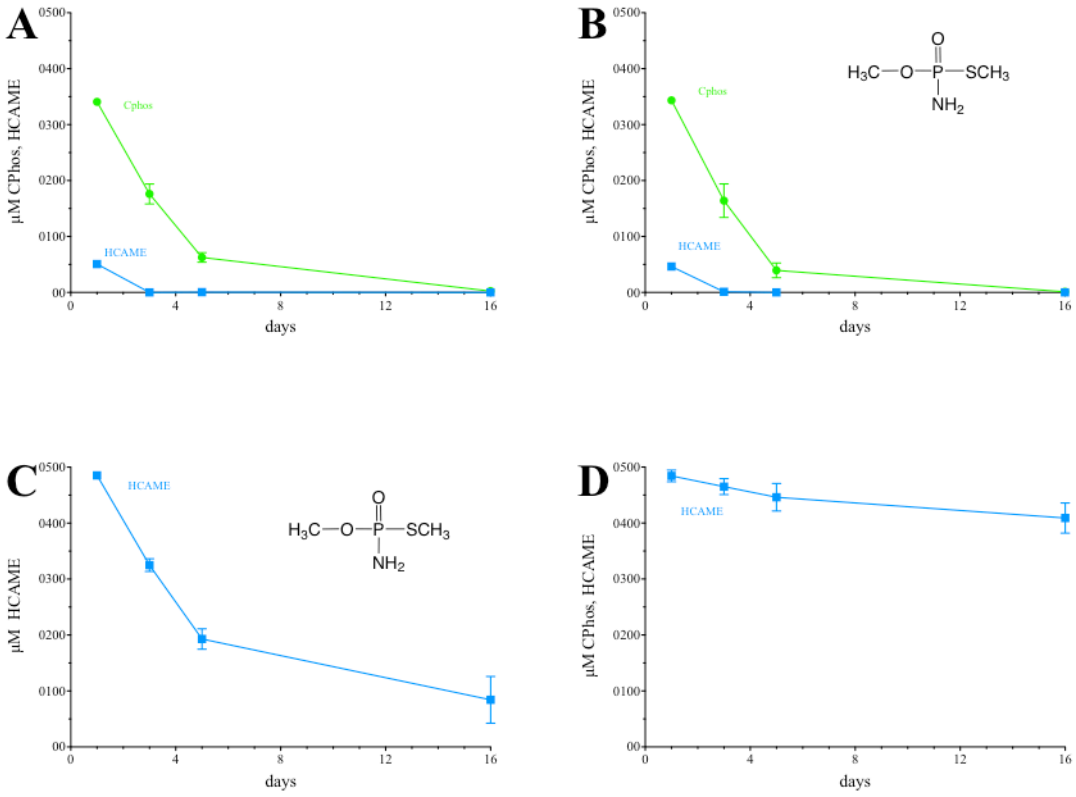


Figure 25. Effect of methamidophos on CP and HCAME degradation. A) CP provided alone, B) CP and methamidophos, C) HCAME and methamidophos, D) HCAME alone. All substrates provided at 500 µM. Bars indicate 1 sd for triplicate cultures.

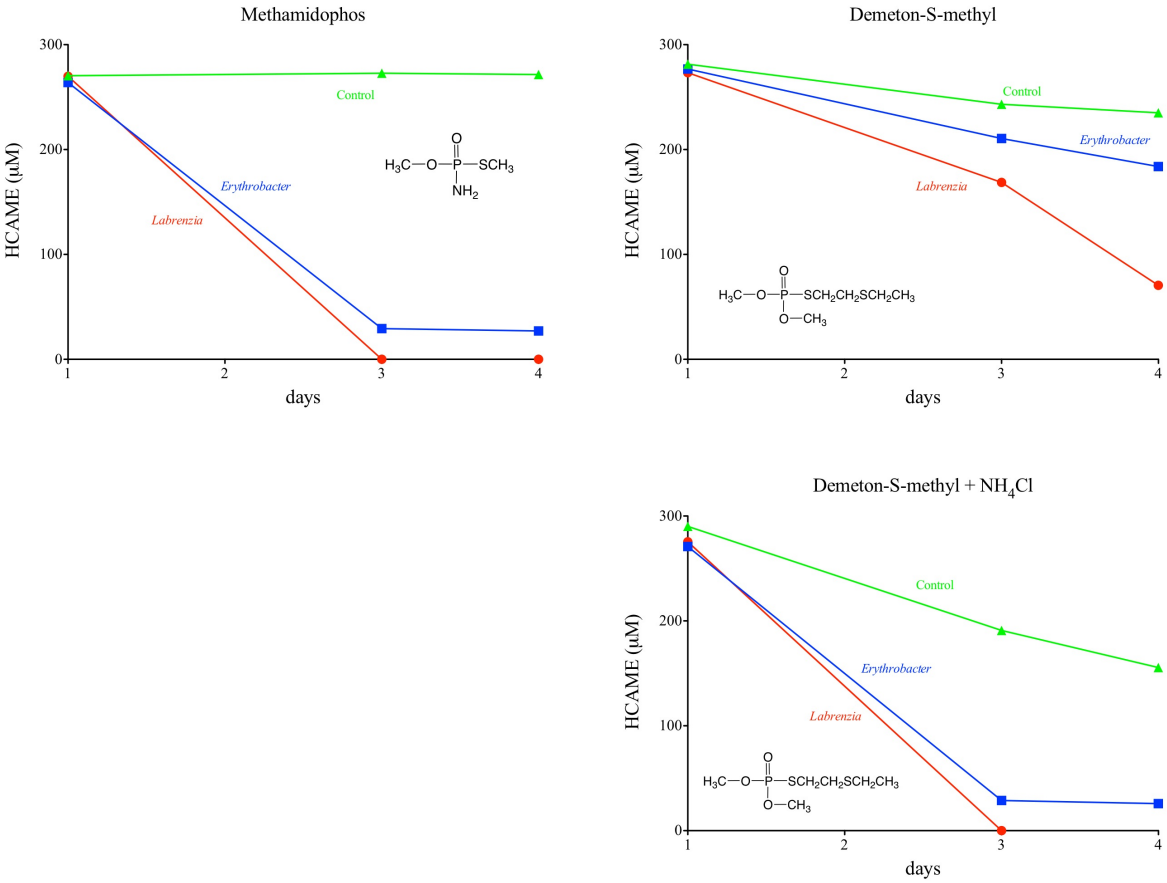


Figure 26. HCAME was degraded when organophosphate pesticides were provided to the isolates as the sole phosphorus source.

Tables

Table 1. Substrate specificities of putative 2-nitroalkane dioxygenases

Substrates	Gene cloned					
	Bphyt4144[a]		Bphyt6745[a]		PA4202[b]	
	[c]NO_2^-	[d]O_2	NO_2^-	O_2	NO_2^-	O_2
3-Nitropropioic acid	-	nd	-	nd	-	nd
Propionate 3-nitronate	+	23.2 ± 0.6	+	28.9 ± 0.4	+	68.2 ± 1.6
2-Nitropropane	-	nd	-	nd	-	nd
Propane 2-nitronate	-	nd	-	nd	+	nd

a. From *Burkholderia phytofirmans* PsJN

b. From *Pseudomonas aeruginosa* PAO1

c. Nitrite release

d. Specific activity (μmol O_2/mg protein/min), nd = not detected

Table 2. Growth responses of JS670 and mutant to analogs of NPE.

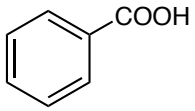
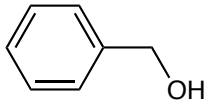
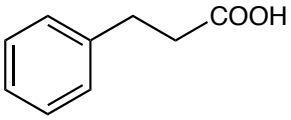
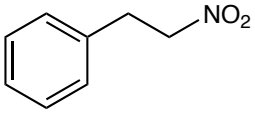
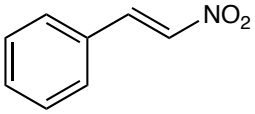
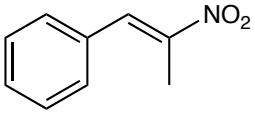
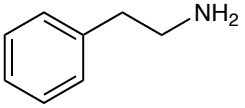
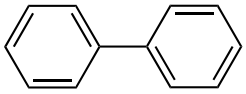
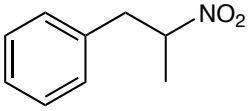
Compound	Structure	*Burkholderia* sp. JS670	*Burkholderia* sp. JS670 ΔNPE dioxygenase
Benzoic Acid		+	+
Benzyl Alcohol		+	+
Cinnamic Acid		-	-
1-Nitro-2-phenylethane		+	-
Trans-β-nitrostyrene		+	-
2-Methyl-β-nitrostyrene		+	-
Phenylethylamine		+	-
Biphenyl		+	+
2-Nitro-1-phenylpropane		+	-

Table 3. Activity of 4-nitrobenzoate reductase in cell extracts of JS674.

4-Nitrobenzoate Reductase Activity in JS674		
Growth Substrate	**Cofactor**	**Specific Activity (nmol/min·mg)**
Chloramphenicol	NADPH	135 ± 18
Chloramphenicol	NADH	72 ± 1
Glucose	NADPH	not detected
Pyruvate	NADPH	not detected