REPORT DOCUMENTATION PAGE

AFRL-SR-BL-TR-98-
0346

Public reporting burden for this collection of information is estimated to average 1 hour per response, inclu... gathering and maintaining the data needed, and completing and reviewing the collection of information. Se... collection of information, including suggestions for reducing this burden, to Washington Headquarters Serv... Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperv...

1. AGENCY USE ONLY (*Leave blank*)

2. REPORT DATE

3. REPORT TYPE AND DATES COVERED
FINAL 15 Jun 94 To 14 Sep 97

4. TITLE AND SUBTITLE
GENETICS AND REGULATION OF NITROAROMATIC HYDROCARBON DEGRADATION

5. FUNDING NUMBERS
F49620-94-1-0258
2312/AS
61102F

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10. SPONSORING/MONITORING AGENCY REPORT NUMBER

19980511 063

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION/AVAILABILITY STATEMENT
Approved for public release;
distribution unlimited.

12b. DISTRIBUTION CODE

13. ABSTRACT (*Maximum 200 words*)
Catabolic pathways for nitroaromatic compound degradation that are initiated by reductive or monooxygenase enzymes are being examined at the molecular level. The gram negative P. fluorescens strain ENV2030 degrades p-nitrophenol oxidatively via hydroquinone. All of the genes for this catabolic pathway have been cloned and their nucleotide sequence determined. Two genes encoding for regulatory proteins have been identified. The P. fluorescents ENV2030 cloned genes have been shown to hybridize with genomic DNA from other p-nitrophenol degrading strains (P. putida JS444 and moraxella species strain 1A). The genes for p-nitrophenol degradation have been cloned from P. putida JS444. A comparison of the genes from ENV2030 and JS444 shows that although they are 80-90% identical a gene for a key regulatory protein is missing. This explains the differences seen in how the metabolism of p-nitrophenol is regulated in the two strains. The gram negative P. pickettii strain YH105 degrades p-nitrobenzoate and p-aminobenzoate reductively via protocatechuate. The genes for the degradation of p-nitrobenzoate ahve been cloned and their nucleotide sequence determined. A regulatory gene has been identified. The gram negative strains P. syringae HY101 and P. putida YH102 also degrade p-nitrobenzoate by a reductive route but genomic

14. SUBJECT TERMS

15. NUMBER OF PAGES

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
(U)	(U)	(U)	(UL)

DTIC QUALITY INSPECTED 3

DNA from these two strains does not cross hybridize to the genes cloned from p. pickettii YH105, indicating diversity in the gene sequences. The genes for p-nitrobenzoate degradation have been cloned from YH101 and HY102 and shown to be very similar to each other. The cloned YH102 genes have been sequenced and the results show that although the HY102 and HY105 genes are 50-60% identical they are organized quite differently.

Genetics and Regulation of Nitroaromatic Hydrocarbon Degradation
Grant No. F49620-94-1-0258

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Progress Report 01 Aug 96 - 31 Oct 97 (Final)

Prepared for
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Objectives

The present research is to develop fundamental information dealing with the microbial degradation of various nitroaromatic compounds. Special emphasis will be placed on analyzing the genes involved in the initial steps of the catabolic pathway and determining the mechanisms of their regulation. Recombinant constructs will be constructed that express the genes of interest to analyze substrate range and product formation.

Status of Effort (Abstract)

Catabolic pathways for nitroaromatic compound degradation that are initiated by reductive or monooxygenase enzymes are being examined at the molecular level. The gram negative *P. fluorescens* strain ENV2030 degrades *p*-nitrophenol oxidatively via hydroquinone. All of the genes for this catabolic pathway have been cloned and their nucleotide sequence determined. Two genes encoding for regulatory proteins have been identified. The *P. fluorescens* ENV2030 cloned genes have been shown to hybridize with genomic DNA from other *p*-nitrophenol degrading strains (*P. putida* JS444 and *Moraxella* species strain 1A). The genes for *p*-nitrophenol degradation have been cloned from *P. putida* JS444. A comparison of the genes from ENV2030 and JS444 shows that although they are 80-90% identical a gene for a key regulatory protein is missing. This explains the differences seen in how the metabolism of *p*-nitrophenol is regulated in the two strains. The gram negative *P. pickettii* strain YH105 degrades *p*-nitrobenzoate and *p*-aminobenzoate reductively via protocatechuate. The genes for the degradation of *p*-nitrobenzoate have been cloned and their nucleotide sequence determined. A regulatory gene has been identified. The gram negative strains *P. syringae* YH101 and *P. putida* YH102 also degrade *p*-nitrobenzoate by a reductive route but genomic DNA from these two strains does not cross hybridize to the genes cloned from *P. pickettii* YH105, indicating diversity in the gene sequences. The genes for *p*-nitrobenzoate degradation have been cloned from YH101 and YH102 and shown to be very similar to each other. The cloned YH102 genes have been sequenced and the results show that although the YH102 and YH105 genes are 50-60% identical they are organized quite differently.

Accomplishments:

1) Molecular analysis of *p*-nitrophenol degradation by gram negative organisms

Several gram negative bacterial strains that degrade *p*-nitrophenol were acquired. These include *P. fluorescens* strain ENV2030 from Envirogen, Inc., (Lawrenceville, New Jersey) and *P. putida* strain JS444 and *Moraxella* sp. strain 1A from Dr. Jim Spain (AL/EQC-OL, Tyndall AFB). All of these strains degrade *p*-nitrophenol through the pathway shown in Figure 1. Briefly, this pathway involves monooxygenase attack of the aromatic ring with the release of the nitro group as nitrite and the formation of benzoquinone. The latter compound is subsequently reduced to form hydroquinone which is the substrate for ring cleavage. Further enzymatic reactions lead to TCA cycle intermediates. The initial choice for a molecular investigation of this catabolic pathway was *P. fluorescens* strain ENV2030. The genes for *p*-nitrophenol

degradation were cloned from this strain by tagging the genes with a transposon, cloning the transposon insertion from the strain, and using DNA flanking the transposon insertion as a probe to screen a cosmid library. Complementation experiments were performed with subclones and chemically- and transposon-derived mutants to locate the genes on the clones. Data from these experiments implicated a 17.8 kb *Not*I fragment as containing the desired genes. The nucleotide sequence of this entire fragment was determined. Open reading frames were correlated with observed mutant complementation data and enzymatic activities expressed in *E. coli*. An analysis of this region is shown in Figure 1. Two regulatory genes were identified based on the deduced amino acid sequence of the encoded proteins. The data from the transposon mutagenesis experiments indicate that PnpR is involved in positive regulation of *pnpA*. PnpS, on the other hand, is involved in positive regulation of *pnpDEC*. Currently additional experiments are being performed to determine the inducers for each of these regulatory circuits.

One question that was posed in the initial proposal was how widespread the genes for *p*-nitrophenol degradation are in the environment and whether (and how much) diversity exists for the individual enzymes and regulatory proteins. Now that a clone was obtained that contained the genes for *p*-nitrophenol degradation from *P. fluorescens* ENV2030 it was possible to begin answering that question. The *Not*I fragment described above was used to determine if two other well known *p*-nitrophenol degrading strains (*P. putida* JS444 and *Moraxella* sp. strain 1A from Dr. Jim Spain) contained similar genes. The Southern hybridization experiments indicate that both of these strains contain a large *Not*I fragment that cross hybridizes with the corresponding fragment from *P. fluorescens* ENV2030. This region has been cloned from *P. putida* JS444 and partially sequenced. The data indicate that while the genes for *p*-nitrophenol degradation in JS444 and ENV2030 are arranged into similar operons (see Figure 1) and are 80-90% the same JS444 is missing *pnpR*. This may explain the fact that JS444 is induced by the buildup of a compound in the culture fluid. Experiments are underway to determine whether the two operons in JS444 (*pnpA* and *pnpDEC*) are both induced by hydroquinone (or another downstream metabolite) by the action of PnpS. This would mean that an organism such as ENV2030 would be much more efficient at degrading *p*-nitrophenol (than JS444) due to the fact that it contains a specific regulatory protein responding to *p*-nitrophenol.

2) Molecular analysis of *p*-nitrophenol degradation by gram positive organisms

It has now been determined by a number of groups that gram positive organisms degrade *p*-nitrophenol through 1,2,4-trihydroxybenzene rather than through hydroquinone. Dr. Spain generously sent several *Arthrobacter* strains for molecular analysis. A cosmid library has been constructed for one of the strains using partially *Mbo*I digested genomic DNA and the cosmid cloning vector pHC79. Attempts to locate the genes in the library by expression in *E. coli* were unsuccessful. The enzyme responsible for cleaving 1,2,4-dihydroxybenzene has been purified from the strain and is being subjected to N-terminal sequencing. The N-terminal sequence will be used to design PCR primers so that a corresponding region of the genome can be obtained to use as a probe to locate a cosmid clone containing this gene. Assuming that the genes for *p*-nitrophenol degradation are linked in this strain the genes for the other steps in the pathway should be on the same cosmid clone.

3) Molecular analysis of *p*-nitrobenzoate degradation

Three strains that degrade p-nitrobenzoate have been under investigation. Most of the research has been performed with *P. pickettii* YH105 and recently work has begun with *P. syringae* YH101 and *P. putida* YH102. Initial experiments with these three strains showed that growth on *p*-nitrobenzoate results in the accumulation of ammonia in the culture medium. This suggests that all three strains degrade *p*-nitrobenzoate via a reductive route as has been shown for other microorganisms. Further experiments involving enzyme assays and metabolite analyses demonstrated that YH105 degrades *p*-nitrobenzoate through *p*-hydroxylaminobenzoate and protocatechuate (Figure 2).

In order to investigate the ability of YH105 strain to degrade *p*-nitrobenzoate in more detail at the molecular level gene cloning studies were initiated. The genes were initially isolated from a cosmid library and located by subcloning and expression studies. These experiments suggested that a 2.2 kb *SalI* fragment contained the genes coding for *p*-nitrobenzoate reductase and hydroxylaminolyase, the two steps in this branch of the catabolic pathway. This region was then sequenced. Genes for both enzymes were identified. A cartoon of the nucleotide sequence is shown in Figure 2. The open reading frames for the reductase and the hydroxylaminolyase were identified through previous enzymatic activities generated from subclones. Additionally the gene for the reductase was located through purifying the protein and determining its N-terminal sequence. Analysis of the nucleotide sequence also revealed the presence of a putative regulatory gene transcribed in the opposite direction from the structural genes (Figure 2).

One question that we wish to answer is how much diversity exists for the individual enzymes and regulatory proteins for *p*-nitrobenzoate degradation. Now that a clone was obtained that contained the genes for *p*-nitrobenzoate degradation from *P. pickettii* YH105 it was possible to begin answering that question. Several bacterial strains were obtained that degrade *p*-nitrobenzoate by a reductive route. The *p*-nitrobenzoate degrading strains *P. syringae* YH101 and *P. putida* YH102 were isolated by Yarek Hrywna in our laboratory from the same location (and at the same time) as *P. pickettii* YH105. *Pseudomonas* species strain 4NT was obtained from Dr. Jim Spain and degrades *p*-nitrotoluene through *p*-nitrobenzoate. *C. acidovorans* NBA-10 was obtained from Dr. Jan DeBont (Wageningen University, The Netherlands) and grows on *p*-nitrobenzoate. Southern blots with the genes for *p*-nitrobenzoate degradation (2.2 kb *SalI* fragment) from *P. pickettii* YH105 as the probe do not show any hybridization to genomic DNA prepared from the other four strains. This indicates that there is significant diversity among the genes for *p*-nitrobenzoate degradation from these five strains (or at least that there are two classes of genes).

In order to explore the diversity of the genes for *p*-nitrobenzoate degradation among these five strains it was decided to clone the genes from both *P. syringae* YH101 and *P. putida* YH102. This was accomplished by constructing a cosmid library and screening the resulting *E. coli* colonies for the ability to convert *p*-nitrobenzoate to protocatechuate by a colorimetric plate assay technique. Cosmid clones that are able to catalyze this conversion were obtained from each strain. Initially, the cosmid clone obtained from *P. putida* YH102 was the focus of our investigation. Several subclones were obtained and their ability to catalyze the conversion of

p-nitrobenzoate to protocatechuate was determined. The smallest region able to perform this conversion is a 3.2 kb *Eco*RI-*Bam*HI fragment of DNA. Sequencing of this region located two open reading frames, one coding for a regulatory protein and the other coding for the hydroxylaminolyase. These genes are 50-60% identical to the corresponding genes found in YH105. A gene for a reductase was not located on the clone. indicating a different operonic structure for the genes for *p*-nitrobenzoate degradation. A knockout mutant of the regulatory gene was constructed and shown to not metabolize *p*-nitrobenzoate at all. This means that although the gene (*pnpA*) for the reductase is located elsewhere in the genome it is still regulated by the same regulatory protein as that found next to *pnpB*, the gene for the second step in the pathway. A knockout mutant of *pnpB* results in accumulation of *p*-hydroxylaminobenzoate, as expected. Further experiments are underway to locate the gene for the reductase. A comparison of the gene organizations of YH102 and YH105 is shown in Figure 2.

Significant homology was seen in Southern hybridization experiments using the *P. putida* YH102 genes described above and the cosmid clone containing the genes for *p*-nitrobenzoate degradation obtained from *P syringae* YH101. This indicates that the genes for *p*-nitrobenzoate degradation in *P. putida* YH102 and *P. syringae* YH101 are closely related. Subcloning and nucleotide sequencing are underway for the YH101 clones in order to determine how closely related the genes actually are.

Personnel Supported 8/94-9/97:

Gerben J. Zylstra	Assistant (now Associate) Professor
Asha Yabannavar	Postdoctoral Associate (8/94-11/95) funded by the AFOSR grant
Lisa Newman	Postdoctoral Associate (3/96-present) funded by the AFOSR grant
Sang-Weon Bang	Graduate Student (8/94-8/97) funded by the AFOSR grant
Lynda Callicotte	Graduate Student (9/94-present) funded by an AASERT supplement
Michael Jackson	Graduate Student (9/97-present) funded by an AASERT supplement
Danielle Cherdak	Undergraduate Student funded by the AFOSR grant
Paul Washart	Undergraduate Student funded by the AFOSR grant
Latika Vasudeva	High School Student funded by the AFOSR grant
Kwok-Lam Ho	Undergraduate Student funded by the AFOSR grant
Betsy Merz	Undergraduate Student funded by the AFOSR grant
Imelda Harjono	Undergraduate Student funded by the AFOSR grant

Publications:

Bang, S.-W. 1997. Molecular analysis of *p*-nitrophenol degradation by *Pseudomonas* sp. strain ENV2030. Ph.D. Thesis, Rutgers University.

Newman, L. M., and G. J. Zylstra. 1997. Cloning and characterization of genes involved in 4-nitrobenzoate degradation from *P. putida* YH102. Abstr. Am. Soc. Microbiol., Q-341, p. 512.

Bang, S.-W., and G. J. Zylstra. 1997. Cloning and sequencing of the hydroquinone 1,2-dioxygenase, 2-hydroxymuconic semialdehyde dehydrogenase, and maleylacetate

reductase genes from *Pseudomonas fluorescens* ENV2030. Abstr. Am. Soc. Microbiol., Q-383, p. 519.

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Bang, S.-W., and G. J. Zylstra. 1996. Cloning and characterization of the genes involved in *p*-nitrophenol degradation by *Pseudomonas fluorescens* ENV2030. Abstr. Am. Soc. Microbiol., Q166, p. 414.

Yabannavar, A., and G. J. Zylstra. 1995. Cloning and characterization of the genes for *p*-nitrobenzoate degradation from *Pseudomonas pickettii* YH105. Abstr. Am. Soc. Microbiol., Q175, p. 430.

Yabannavar, A. V., and G. J. Zylstra. 1995. Cloning and characterization of the *P. pickettii* YH105 genes for *p*-nitrobenzoate degradation. Appl. Environ. Microbiol. 61:4282-4290.

Interactions/Transitions:

A clone containing the *p*-nitrophenol oxygenase was sent to Wilfred Chen, Chemical Engineering, UC Riverside, to use in biomonitoring experiments.

A clone containing all of the genes for *p*-nitrophenol degradation was sent to Jay D. Keasling, Department of Chemical Engineering, UC Berkeley to use in biodegradative studies.

A trip was made to Dr. David Gibson's laboratory at the University of Iowa August 24 to 27, 1996. This meeting was held between Dr. Gibson's group (D. Gibson and R. Parales), Dr. Zylstra's group (G. Zylstra and A. Goyal), and Dr. Spain's group (J. Spain and C. Sommerville of Tyndall AFB) to discuss comparative analysis of genes (and enzymes) involved in nitroaromatic compound degradation. An outline of a review paper to be submitted to *Microbiological Reviews* was constructed. Discussions were also held on topics of mutual interest in nitroaromatic compound degradation and on ways that the three laboratories could perhaps interact and help each other with their AFOSR-funded projects.

Rutgers University is the recipient of an Advanced Research Projects Agency University Research Initiative (ARPA URI) grant. Ten faculty from the departments of Chemical and Biochemical Engineering, Marine Science, Environmental Science, and Biochemistry and Microbiology are involved in the project. Information on nitroaromatic degradation generated from the AFOSR proposal was distributed at every scientific review board meeting. Dr. Jim Spain of Tyndall AFB attends as an observer. Other Department of Defense attendees included Dr. Ira Skurnick of ARPA and Dr. Eric Eisenstadt and Dr. Anna Palmisano of ONR.

Dr. Jim Spain of Tyndall AFB was invited to Rutgers University to give a seminar on September 27, 1995. (Dr. Zylstra was the organizer of this year's seminar series titled "Microbial Diversity and Bioremediation" and was the host for Dr. Spain). During Dr. Spain's visit several discussions were held with members of the Zylstra laboratory regarding nitroaromatic metabolism.

A collaboration was arranged with Drs. J. Goodall and S. Peretti of the Department of Chemical Engineering at North Carolina State University. These researchers are working on bioreactors for the simultaneous degradation of mixed *m*- and *p*-nitrobenzoate wastes. Currently they are using two different strains of bacteria, one for each compound. We have sent them our clone that contains the genes for *p*-nitrobenzoate degradation so that they can place it into the strain that degrades *m*-nitrobenzoate. In a continuing collaboration we have recently sent them a transposon that we designed containing all of the genes for *p*-nitrobenzoate degradation. This transposon will allow the stable integration of the degradative genes into the host genome in such a way that regulated expression of *p*-nitrobenzoate degradation should be seen. This will allow the construction of a single strain able to degrade both the *meta* and *para* isomers of nitrobenzoate.

Comparative nucleotide and amino acid sequence analyses, GenBank database searching, and dendrogram formation to show relationships between the different amino acid sequences were performed for Dr. Billy Haigler of Tyndall Air Force Base (Dr. Jim Spain's laboratory). This involved an analysis of the genes for dinitrotoluene degradation, specifically focusing on the amino acid sequence of the 4-methyl-5-nitrocatechol monooxygenase enzyme and the nucleotide sequence of the genes for the dinitrotoluene dioxygenase.

Comparative nucleotide sequence analysis was performed for Dr. John Davidson of Tyndall Air Force base (Dr. Jim Spain's laboratory) to determine if there is any sequence-based relationship between the genes for the reductive degradation of *p*-nitrobenzoate and the reductive degradation of nitrobenzene.

A collaboration was arranged with Dr. Jan deBont of Wageningen University in The Netherlands. Dr. deBont is funded by industry to investigate the use of biodegradative genes to construct certain catechol compounds (nitro-substituted and otherwise) from chemical feedstocks (nitro-substituted and otherwise). A graduate student (Jasper Kieboom) from Dr. deBont's laboratory spent three months this fall in Dr. Zylstra's laboratory. The time was partially spent cloning genes for dioxygenases, monooxygenases, and reductases that act on nitroaromatic compounds into solvent resistant strains. These strains will then be tested for their ability to produce oxygenated aromatic compounds valuable to the chemical industry.

A collaboration has been arranged with Dr. Chris Batie of Louisiana State University Medical School. Dr. Batie has purified the *p*-nitrobenzoate reductase and determined its flavin content, cofactor stoichiometry, N-terminal sequence, and molecular weight. These factors enabled us to precisely identify the open reading frame corresponding to this enzyme in the nucleotide sequence.

A collaboration has been arranged with Dr. Charles M. Kenerley of the Department of Plant Pathology and Microbiology at Texas A&M University. Dr. Kenerly will be using our cloned genes for *p*-nitrophenol degradation to clone the similar genes from *Moraxella* sp. strain 1A (obtained from Dr. Jim Spain). Dr. Zylstra will then sequence the clone to determine its similarity to that already obtained from *P. fluorescens* ENV2030.

A collaboration has been arranged with Dr. Larry Hanne of California State University at Chico. He is working with *Arthrobacter* strains that are able to degrade *p*-nitrophenol. We have tested his clones for hybridization to our clones and strains (see text of the report for the results). We have also provided advice on cloning his fragments into broad-host-range plasmids and moving the clones into his *Arthrobacter* strains.

A collaboration has been arranged with Dr. Hemant Purohit of the National Environmental Engineering Research Institute in Nagpur, India. Dr. Purohit has sent us genomic DNA from several *p*-nitrophenol degrading strains to test for hybridization to our clones.

The genes for the reductive degradation of *p*-nitrobenzoate have been sent to Dr. Carmen Michan of the Biochemistry Department at the Estacion Experimental del Zaidin in Granada, Spain. He is working with strains that degrade *p*-nitrotoluene and *p*-nitrobenzoate through a similar catabolic pathway and wished to know if his strains have the same genes as our *P. pickettii* YH105 strain.

New Discoveries, inventions, or patent disclosures.

No patent or invention disclosures were filed. However, only two monooxygenases involved in nitroaromatic compound degradation have ever been cloned and sequenced. These are 4-nitro-5-methylcatechol monooxygenase (by Dr. Spain's group) and *p*-nitrophenol monooxygenase (described here). In addition, the clones for the reductive degradation of nitroaromatic compounds from strains YH101, YH102, and YH105 are the first instances of cloning genes for this type of catabolic pathway. In the same regard the nucleotide sequence of the genes from YH102 and YH105 represent the first example of determining the nucleotide sequence of this class of genes.

Honors/Awards:

7/97-6/00 Foundation for Microbiology lecturer
9/92-8/97 National Science Foundation Young Investigator Award
10/91-9/93 Department of Energy Environmental Restoration and Waste Management Distinguished Young Faculty Award

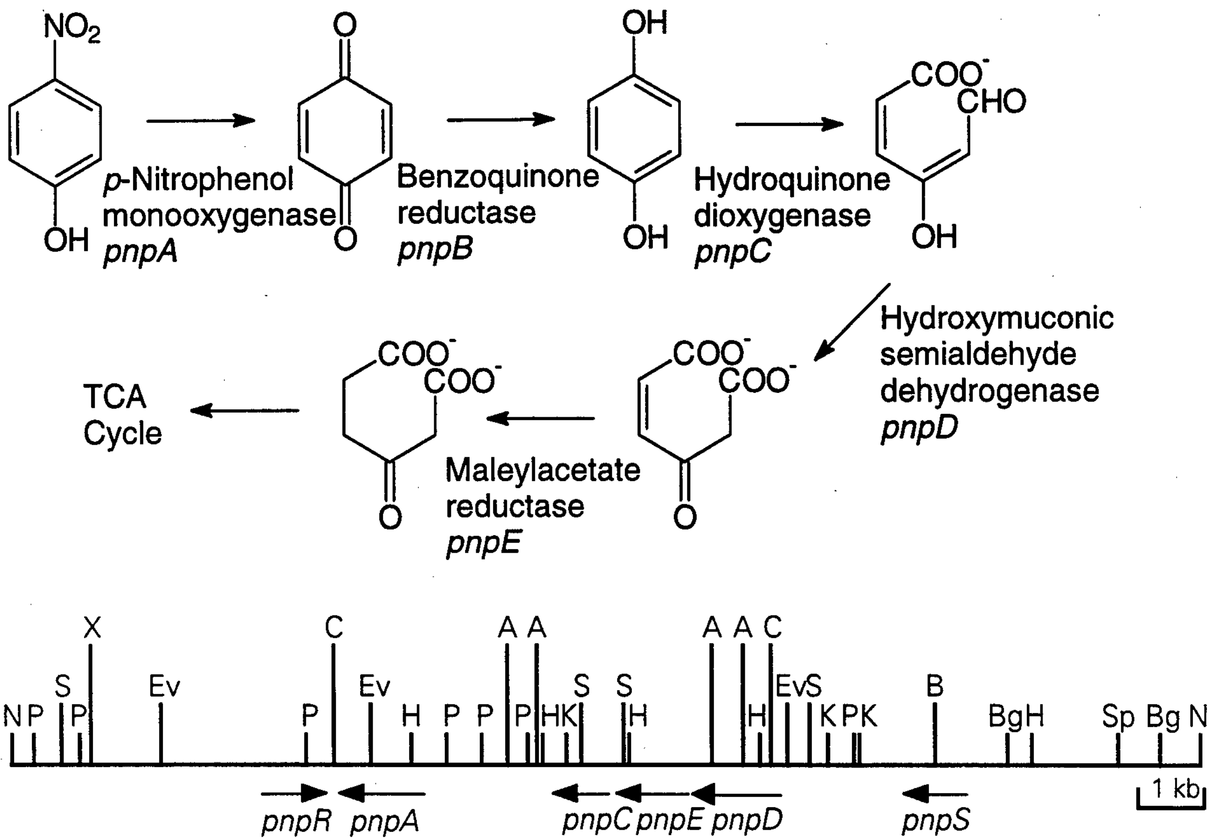

Figure 1. Pathway for the degradation of *p*-nitrophenol. A cartoon of the cloned and sequenced genes for this pathway is also shown. A gene (not shown) suspected to be *pnpB* is located between *pnpD* and *pnpS*.

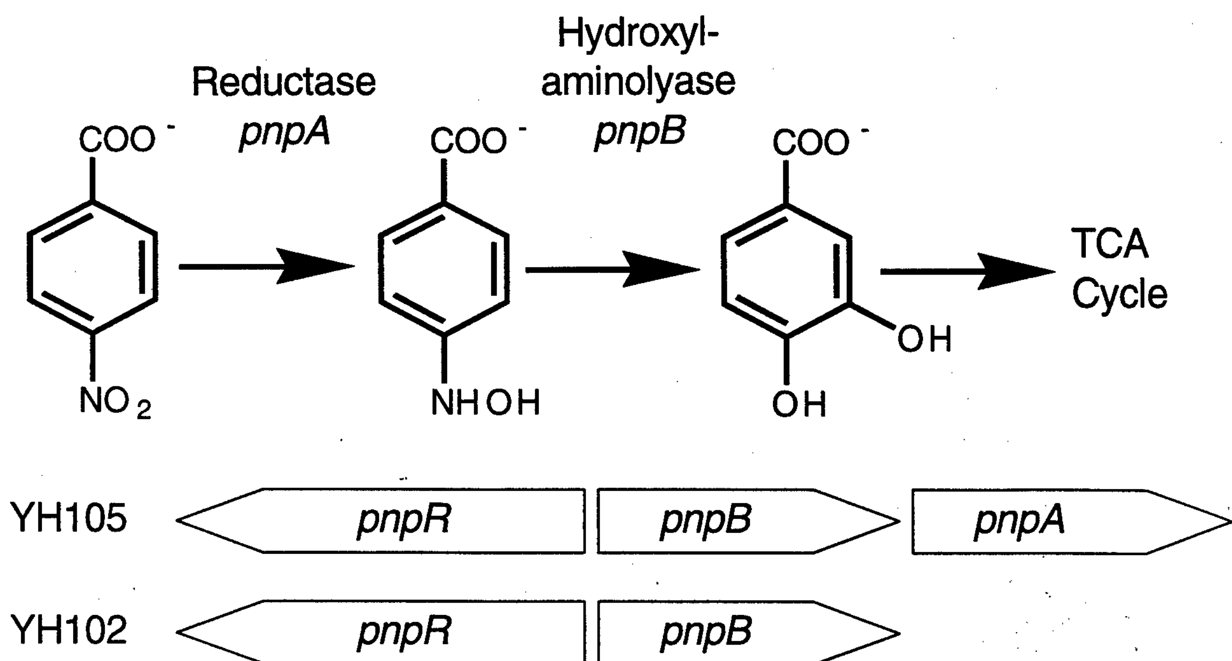

Figure 2. Pathway for the reductive degradation of *p*-nitrobenzoate. A cartoon of the cloned and sequenced genes for this pathway is also shown. Note that while strain YH105 has *pnpB* and *pnpA* adjacent to each other, YH102 does not. *pnpA* in YH102 is located elsewhere in the chromosome.