REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	2. REPORT TYPE	3. DATES COVERED *(From - To)*
4 Mar 99	FINAL	30 SEP 97 - 29 SEP 98

4. TITLE AND SUBTITLE

"MOLECULAR BASIS OF P-NITROPHENOL (PNP) BIODEGRADATION AND ITS APPLICATION IN THE ENVIRONMENT"

5a. CONTRACT NUMBER
F49620-97-1-0538

5b. GRANT NUMBER
AOARD-97-01

5c. PROGRAM ELEMENT NUMBER
61102F

6. AUTHOR(S)
JAIN, RAKESH, K, DR.

5d. PROJECT NUMBER
N/A

5e. TASK NUMBER
N/A

5f. WORK UNIT NUMBER
M/A

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
DR. RAKESH K. JAIN
INSTITUTE OF MICROBIAL TECHNOLOGY
SECTOR 39A
CHANDIGARH - 160036

8. PERFORMING ORGANIZATION REPORT NUMBER
N/A

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)
ASIAN OFFICE OF AEROSPACE RESEARCH & DEVELOPMENT (AOARD)
UNIT 45002
APO AP 96337-5002

10. SPONSOR/MONITOR'S ACRONYM(S)
N/A

11. SPONSOR/MONITOR'S REPORT NUMBER(S)
AOARD-97-01

12. DISTRIBUTION/AVAILABILITY STATEMENT
APPROVED FOR PUBLIC RELEASE; DISTRIBUTION UNLIMITED.

13. SUPPLEMENTARY NOTES

14. ABSTRACT

By taking plasmid samples from the US and India, basic microbial science was related to field application. The distribution of plasmid ecology and gene sequences for a variety of isolates were studied towards an understanding of the genetics and distiributions of microorganisms capable of utilizing PNP as their sole source of carbon, nitrogen, and energy. The studies indicate that 1) the degradation of PNP by several organisms proceeds through an oxidative route, as indicated by the accumulation of nitrite molecules in his cultures, 2) the plasmid responsible for carrying the gene for PNP degradation in certain microbes consists of approximately 50 kilobase pairs, and 3) the same plasmid also encodes resistance to inorganiz zinc ions. Genes were successfully cloned as a basis for further environmental application. As a means of natural attenuation, the development of bioremediation technologies could save millions in addressing contamination sites, with wide application to the remediation of contaminants in soil and groundwater everywhere.

15. SUBJECT TERMS
Molecular basis, p-nitrophenol (PNP) biodegradation, plasmid samples and ecology, oxidative route.

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			MS. JOANNE MAURICE
U	U	U	UU	4	19b. TELEPHONE NUMBER *(Include area code)* 81-3-5410-4409

Final Technical Report

Project Title: Molecular basis of p-nitrophenol (PNP) biodegradation and its application in the environment.

Principle Investigator: Dr. Rakesh K. Jain
Institute of Microbial Technology
Sector 39A
Chandigarh - 160036.

Grant Number: **F49620-97-100538**

Research Objectives:

The proposed research work had the following objectives:

1. To determine the plasmid ecology of PNP degradation in different organisms isolated in India and the US laboratories.
2. Isolation and characterization of the genes encoding PNP degradation for construction of DNA probes.
3. Development of reporter gene system for PNP degradation to identify the PNP/other nitroaromatic degrading organisms/genes in the environment.

Progress of work:

Nitrophenols are an important class of compounds because they are widely used and have been shown to persist in the environment. Since there is very little information available on the degradation of these compounds, it is very important to understand the regulation of degradation of nitrophenols including PNP at the molecular level.

Furthermore, understanding of the distribution and dispersal of PNP degrading genes in the environment is also important as this would provide useful information about the occurrence and nature of the genes.

A total of 14 PNP degrading organisms isolated in the US and Indian laboratories were studied for ecological aspects: JS401, JS402, JS403, JS404, JS405, JS406, JS409, JS411, JS425, JS428, JS443, JS444, RKJ100 and RKJ200. The identification and characterization of these organisms has not been completed so far and is presently underway. Initially the growth and nitrite release assay (1) by these strains on PNP and 4-nitrocatecol (4NC) was carried out. Since some of these organisms also have the ability to utilize or release nitrite from 4NC, a possible intermediate of the PNP-degrading pathway, the growth and nitrite release was also checked on this compound. These results are shown in Table 1.

Since the genes for aromatic hydrocarbon degradation in many cases are plasmid-encoded, attempts were made to check if the plasmids are present in any of the above strains and if they have any role in the degradation pathway. Plasmids were isolated by several methods, however, the method of Anderson and McKay was found to be most suitable (2) and these results are shown in Table 1. All plasmids were large in size

(above 50kb in size, data not shown). Furthermore, based on the morphological and some genetic characteristics, it was concluded that organisms JS403, JS405, JS409 and JS411 are the same (Group I). Similarly, organisms JS401, JS402, JS404 and JS406 are the same (Group II).

In order to check if the plasmids present (as indicated above) have any role in the PNP and/or 4NC degradation, attempts were made to cure these plasmids. Mitomycin C was used as curing agent and three cycles of treatment were performed (3). Dilutions were plated out onto nutrient agar and colonies were picked and patched onto MMagar containing PNP as sole carbon source to score PNP^- derivatives. The ability of PNP^- derivatives to revert to growth on PNP was determined by inoculating approximately 10^{10}-10^{11} cells from a washed, overnight nutrient broth culture onto MMagar containing PNP. The PNP^- derivatives obtained as above were also checked for their growth and release of nitrite molecules on 4-nitrocatecol. The summary of the data thus obtained revealed the results as follows. Since, Group I organisms JS403, JS405, JS409 and JS411 did not show the presence of any plasmid by using different methods, the degradation of PNP and 4NC was not plasmid-encoded as indicated by the isolation of mutant strains. However, it was clearly shown that the degradation of PNP and 4NC was plasmid-encoded in case of organisms belonging to Group II (JS401, JS402, JS404 and JS406), this was confirmed by the isolation and characterization of $PNP^-/4NC^-$ derivatives and release of nitrite molecules by these derivatives. Similarly, based on the studies with the wild-type and mutant derivatives, it was shown that the degradation of PNP and 4NC is plasmid-encoded in case of RKJ100 and RKJ 200. On the other hand, the degradation of the above compounds was found not to be plasmid-encoded in case of JS425, JS443 and JS444.

As one of the objectives of the proposed work, attempts were made to clone some of the genes for PNP degradation from RKJ100/RKJ200. Since the genes for PNP degradation are plasmid-encoded in these cases, the plasmids from these organisms were isolated by the method of Anderson and McKay (2) and purified by CsCl-Etbr density gradient ultracentrifugation. The molecular methods used were as described earlier (4). A broad host-range plasmid vector pLAFR3 (5) was used for cloning purposes. The purified plasmids in both cases were partially digested with restricted enzyme *Eco*R I and were ligated with the *Eco*R I digested vector pLARF3 which carries tetracycline resistant (Tc^R) and lac z (β - galactosidase) genes. The ligated hybrid plasmids were transformed into *E.coli JM109* by electroporation and white, Tc^R colonies were screened. The colonies were then screened by rapid nitrite release assay in order to check the presence of gene(s) encoding PNP oxygenase and/or NC oxygenase. The whole genomic library thus obtained was also transferred into a plasmid-free *Alcaligenes eutrophus JMP222* strain since it is known that many a times degradative genes are not well expressed in *E. coli*. Take together the above results demonstrated that the gene(s) for PNP oxygenase and NC oxygenase have been cloned both from RKJ100 and RKJ200. We are in the process of further characterization of these and other pathway genes.

Conclusions:

It is clear that some important and significant findings regarding nitroaromatics /PNP degradation have been obtained during the above one year project. We were successfully able to examine several-PNP-degrading organisms isolated in the US and India in regard to their plasmid profiles and degradative pathways which provides a basis of the dispersal of these genes in the environment. Some of the genes of the PNP/4NC degradation pathways have also been cloned successfully for further environmental application.

Keeping in mind the progress made, the Principle Investigator (PI) proposes that the funding of the project should continue for another one year. During this period the actual field trials shall be carried out as regard to determine sites in the environment using the DNA probes. These probes would be constructed using the cloned genes as obtained during the course of above work. Furthermore, attempts would also be made to characterize and clone the genes for whole metabolic pathways such as PNP/4NC and other nitroaromatic compounds.

References:

1. Litchfield, M.H. (1968) Analyst 93: 653-659.
2. Anderson, D.G and McKay, L.L. (1983). Appl. Environ. Microbiol. 46: 549-552.
3. Prakash, D., Chauhan, A. And Jain, R.K. (1996). Biochem. Biophys. Res. Commun. 224: 375-381.
4. Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989). Molecular Cloning: A Laboratory Manual 2nd edition. Cold Spring Harbor, NewYork: Cold Spring Harbor Laboratory.
5. Straskawics, B., Dahlbeck, D., Keen, N. And Napoli, C. (1987). Molecular Characterization of cloned avirulence genes from race o and I of *P. Syringae pv. Glycinae*. J. Bacteriol. 160: 5789-5794.

Table 1:

Organism	Growth on PNP	Nitrite release on PNP	Growth on 4NC	Nitrite release on 4NC	Presence of plasmid
JS401	+	+	–	±	+
JS402	+	+	–	±	+
JS403	+	+	–	+	–
JS404	+	+	–	±	+
JS405	+	+	–	+	–
JS406	+	+	–	±	+
JS409	+	+	–	+	–
JS411	+	+	–	+	–
JS425	+	+	–	+	–
JS428	+	+	+	+	+
JS443	+	+	–	+	–
JS444	+	+	–	+	–
RKJ100	+	+	+	+	+
RKJ200	+	+	+	+	+