Plasmid-Encoded Degradation of *p*-Nitrophenol by *Pseudomonas cepacia*

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A *Pseudomonas cepacia* strain RKJ 200 capable of utilising *p*-nitrophenol (PNP+) as the sole source of carbon, nitrogen, and energy was isolated by selective enrichment. The degradation of PNP by this strain proceeds through an oxidative route as indicated by the accumulation of nitrite molecules in the culture medium. Initial studies indicate that the degradation of PNP occurs via hydroquinone as shown by thin layer chromatography and gas chromatography studies; hydroquinone is further degraded via the β-ketoadipate pathway. A plasmid of approximately 50 kilobase pairs was found to be responsible for carrying genes for PNP degradation in this strain. This was based on the facts that the PNP− mutants lacked the plasmid and that the PNP+ phenotype could conjugally be transferred. In addition, the same plasmid also encoded resistance to inorganic zinc ions.   © 1996 Academic Press, Inc.

Rapid industrialisation in the world today has made pollution a matter of prime concern. Among others, nitroaromatic compounds are widely distributed in the environment. *p*-Nitrophenol (PNP), in particular, has been extensively used in the manufacture of pharmaceuticals, pesticides, plasticizers, azo dyes, explosives etc. (10,11,22,30). It is also a major metabolite in the hydrolysis of organophosphates such as parathion(o,o-diethyl o-*p*-nitrophenyl phosphorothiote) and methylparathion (o,o-dimethyl o-*p*-nitrophenyl phosphorothionate) which have been commonly used as pesticides/herbicides; as a result, it can build up in the soil (23). *p*-Nitrophenol has also been listed as a priority pollutant by the United States Environmental Protection Agency(6).

Nitroaromatic compounds are either simply transformed to dead end products (12), which many a times prove to be more toxic than the parent compounds, or they may actually utilise the nitroaromatic compounds as a carbon and/or nitrogen source. Bacteria have the ability to oxidatively remove the nitro groups from *o*-nitrophenol (34), *m*-nitrophenol (34) and *p*-nitrophenol (4,11,15,30).

Although there are some reports on the biochemical steps involved in PNP degradation, there are virtually no reports available which may form genetic basis of PNP degradation. Only recently, Hanne et al., (11) suggested the possible involvement of a plasmid in PNP degradation in an *Arthrobacter* sp. but it was not proven. It is important to understand the genetic basis of PNP degradation to not only understand more about the structure, organization and regulation of the genes involved but also to construct/design effective recombinant microbes for bioremediation of contaminated environments. In the present communication, we report the isolation of a *Pseudomonas cepacia* strain RKJ 200 which is capable of utilising PNP as sole carbon, nitrogen and energy source. Our results, for the first time, clearly demonstrate that the genes for the whole PNP degradative pathway are encoded on a transmissible plasmid in strain RKJ 200.

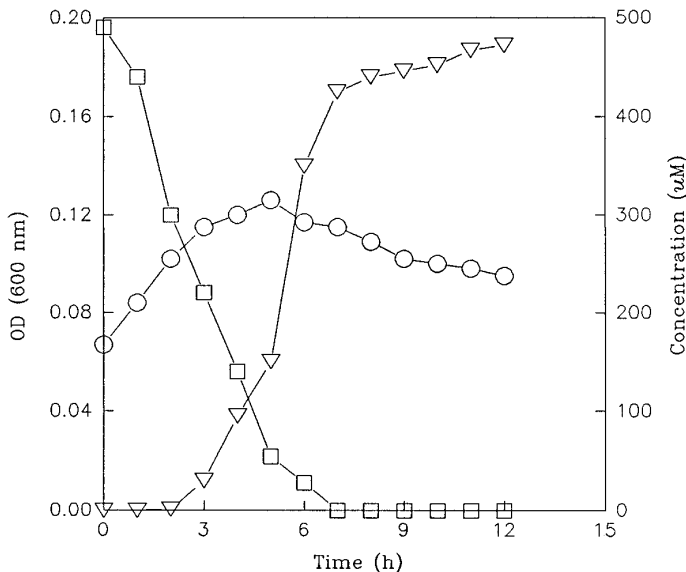[1] Corresponding author. Fax: 0091 172 690585, 690632. E-mail: rkj@imtech.ernet.in.

FIG. 1. Degradation of PNP by *P. cepacia* strain RKJ 200 when grown on PNP as the sole carbon and nitrogen source; strain RKJ 200 rapidly metabolized PNP with concomitant release of nitrite. The inoculum used here was taken from cells of strain RKJ 200 grown overnight in PNP. ○, The optical density (OD) of culture; □, PNP concentration; ▽, nitrite concentration.

MATERIALS AND METHODS

The soil samples from Assam agricultural fields, India, which were sprayed with the pesticide parathion, were used as the inoculum into minimal medium (MM) supplemented with PNP (0.5mM final concentration) as the sole carbon source. Such enriched cultures were incubated at 30°C with shaking (150 rpm) until growth was observed. The yellow colour of PNP changed to colourless indicating that it was being utilised by the organism. Following three subcultures, samples were plated out onto MM agar with PNP as sole carbon source.

Minimal Medium used for growth of strain RKJ 200 had the following composition (per litre): Na_2HPO_4, 4.0g; KH_2PO_4, 2.0g; $MgSO_4$, 0.8g; $(NH_4)_2SO_4$, 0.8g; Yeast extract, 0.02g; trace element solution, 1.0 ml. The trace element solution contained (per litre) (19): $Al(OH)_3$, 0.1g; $SnCl_2$, 0.05g; KI, 0.05g; LiCl, 0.05g; $MnSO_4$ $4H_2O$, 0.08g; H_3BO_3, 0.5g; $ZnSO_4 \cdot 7H_2O$, 0.1g; $CoCl_2 \cdot 6H_2O$, 0.1g; $NiSO_4 \cdot 6H_2O$, 0.1g; $BaCl_2$, 0.05g; $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 0.05g. The pH of the medium was adjusted to 7.0 and agarose (1.5%) was added for solidification of the medium. PNP was added as the sterilized solution to a final concentration of 0.5mM after autoclaving the medium. Following this exercise, one efficient PNP degrading organism, designated as strain RKJ 200, was chosen for further studies. The identification of strain RKJ 200 was carried out by biochemical tests using the Bergeys Manual of Systematic Bacteriology (vol. 1, 1984). Growth on PNP in liquid cultures was monitored by reading absorbance at 600 nm. PNP depletion assay (7) and nitrite release assay (20) were performed as described. Nitrite concentrations were determined by comparison of values with those of a standard calibration curve prepared using sodium nitrite.

The derivatives which were incapable of utilising PNP as sole carbon source were obtained spontaneously or by treatment with mitomycin C. Strain RKJ 200 was grown for 10 cycles in NB for spontaneous mutation studies whereas the organism was grown for 3 cycles in presence of mitomycin C (1.5μg/ml) for induced mutation studies. Dilutions were plated out onto nutrient agar and colonies were picked and patched onto MM agar 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 MM agar containing PNP. Plasmid DNA isolations were carried out as described previously (1,2,18,24,25,26,33), but the method of O'Sullivan and Klaenhammer (24) was found to be most appropriate and was routinely used. Purification of DNA by Cesium Chloride-Ethidium Bromide (CsCl-EtBr) density gradients was carried out as described earlier (27). Restriction endonuclease digestion was performed using enzymes *Hin*d III, *Pst* I and *Pvu* II according to the instructions supplied by the manufacturer (Boehringer, Mannheim, Germany). The conditions for restriction endonuclease digestion and electrophoresis were as described by Jain et al. (14). Conjugation experiments were performed by triparental
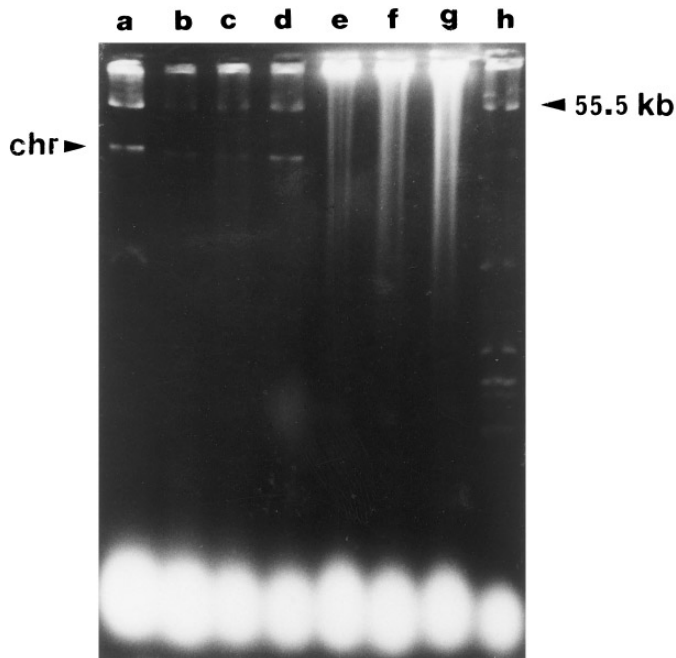
FIG. 2. Agarose gel electrophoresis of plasmid(s) isolated from *P. cepacia* strain RKJ 200: Lanes a, strain RKJ 200; b–d, PNP$^+$, Zn^r transconjugants; e, spontaneous PNP-derivative; f–g, mitomycin C treated PNP$^-$ derivatives; h, *E. coli* V517 as standard plasmid size marker. Chr, chromosome.

mating using the helper plasmid pRK2013 (8) as described earlier (14). Heavy metal resistance of the wild-type strain and its PNP$^-$ derivatives was determined as described by Bopp et al. (3) by inoculating a loopful of overnight nutrient broth culture onto freshly prepared nutrient agar plates containing 250 μg/ml of the appropriate heavy metal salts.

Thin layer chromatography (TLC) and gas chromatography (GC) were performed in order to determine the degradation pathway of PNP in strain RKJ 200. 500 ml of MM containing PNP was inoculated with strain RKJ 200 and the culture was grown with shaking. At different time intervals, 50 ml of the culture was centrifuged and the supernatant was extracted by diethyl ether as described earlier (17). These ether extracts were dried using sodium sulphate and the solvent was dried under vacuum at reduced pressure using a rotary evaporator and finally dissolved in 2 ml of methanol. For TLC studies, 20μl of the above samples were spotted onto precoated silica gel aluminium plates (Merck, Germany). The solvent system used for the seperation of metabolites consisted of toluene/ethyl acetate/acetic acid (60:30:5 v/v) (28). The samples were run for 3 h, dried at room temperature for 5 min and sprayed with Folins reagent and subsequently exposed to ammonia vapours for visualization. *Ortho* or *para* diphenols give an immediate blue colouration, whereas monophenols show up only after exposure to ammonia (17). Gas chromatograph from Hewlett-Packard series II 5890 fitted with an HP I (cross-linked Methyl silicone gum) column (25m × 0.20mm × 0.33μm) was used for gas chromatography. One microlitre of samples were analysed with nitrogen as the carrier gas and oven temperature at 200°C. Peaks were detected by a flame ionization detector. Standard PNP and hydroquinone (10 mg/ml solution in methanol) were also analysed for comparison studies.

RESULTS AND DISCUSSION

Strain RKJ 200 was a gram negative, small rods, oxidase positive, catalase positive, aerobic and motile organism. Growth was observed at 41°C but not at 4°C. It grew on MacConkey agar and utilised citrate. Casein was hydrolysed and nitrate was reduced to nitrite but starch was not hydrolysed, no indole was produced and gelatin was not liquefied. It was capable of growth on protocatechuric acid as sole carbon source and arginine and ornithine were decarboxylased. This organism produced acid from the following sugars: dextrose, sucrose, fructose, lactose, maltose, trehalose, raffinose, mannitol, xylose, salicin, arabinose and sorbitol.
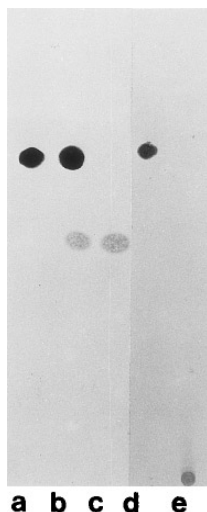
FIG. 3. Thin layer chromatographic analysis of *P. cepacia* strain RKJ 200: Lanes a, PNP as the standard; b, sample drawn at 4 h growth interval; c, hydroquinone as the standard; d, sample drawn at 8 h growth interval; e, sample drawn after overnight growth on PNP.

On the above basis, strain RKJ 200 was identified as a *Pseudomons cepacia* species. Strain RKJ 200 is capable of utilising PNP not only as the carbon and energy source but also as nitrogen source since absence of a nitrogen source in the growth medium did not affect its growth. Strain RKJ 200 was also capable of growth on hydroquinone but not an 4-nitrocatechol or 1,2,4-benzenetriol. It was found that cells of strain RKJ 200 accumulated nitrite in the medium when grown on PNP suggesting the involvement of an initial oxidative step in the degradation of PNP. Further, stoichiometric amount of nitrite was released into the medium concomitant with the degradation of PNP (Fig. 1).

The involvement of catabolic plasmid(s) in the degradation of a number of organic compounds has been shown previously (5,9,16,21). To check if a plasmid(s) is present in strain RKJ 200 that could be involved in PNP degradation, attempts were made to isolate plasmid(s) from this strain. A plasmid of app. 50 kb in size was found to be present using the method of O'Sullivan and Klaenhammer (24) with some minor modifications; *E. coli* strain V517 was used as standard marker plasmids (Fig. 2). Another smaller plasmid of app. 5 kb in size was also detected in strain RKJ 200 which was found to be a cryptic plasmid (Fig. 2; please see below). CsCl-EtBr purified DNA preparations from strain RKJ 200 were digested with enzymes *Hin*dIII, *Pst*I and *Pvu*II to confirm the size of the plasmid(s) (data not shown).

Attempts were made to obtain PNP$^-$ derivatives to determine the role of the plasmid(s) present in strain RKJ 200. The spontaneous loss of PNP$^+$ phenotype was app. 1% which was increased to 3% when mitomycin C was present during growth. When such spontaneously or mitomycin C derived PNP$^-$ derivatives were checked for the presence of plasmid(s) by different methods (please see Materials and Methods), the plasmid of app. 50 kb was absent from them (Fig. 2) indicating the involvement of this plasmid in PNP degradation. Conjugation studies were also carried out in an attempt to transfer the PNP$^+$ phenotype to other strains; *P. putida* PaW 340 (DSM 2112, PNP$^-$, Str^r, Trp$^-$) was used as the recipient strain. Following triparental plate matings, transcojugants were obtained at a frequency of app. 10^{-6} on the selection medium which contained PNP, streptomycin (200 μg/ml) and tryptophan (20 μg/ml). When plasmid DNA was isolated from such PNP$^+$, Str^r, Trp$^-$ tranconjugants, they carried the same plasmid of app. 50 kb as was present in the wild-
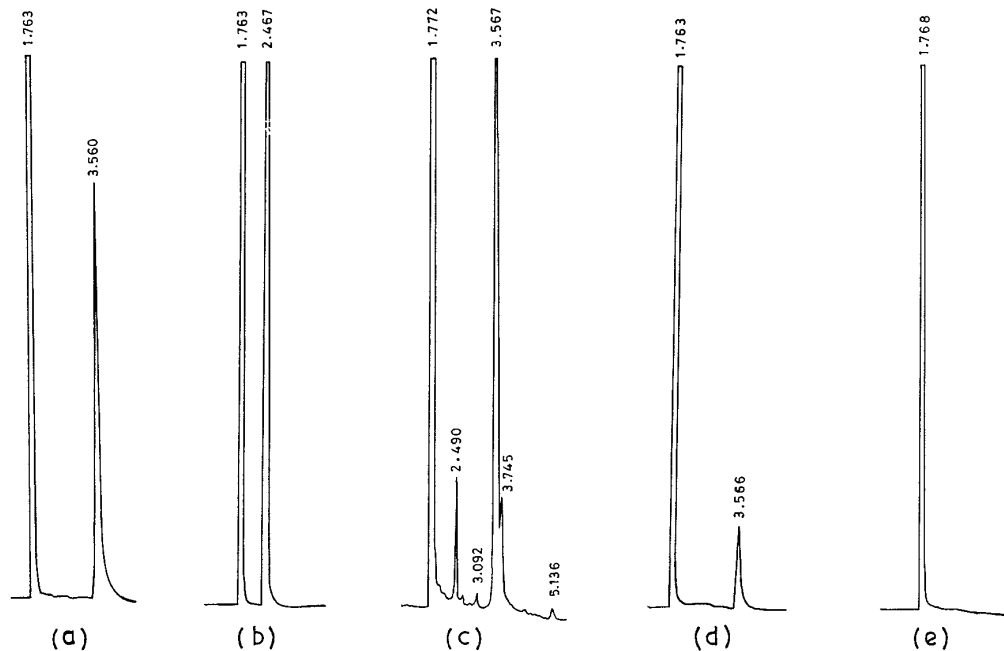
FIG. 4. Gas chromatographic analysis of *P. cepacia* strain RKJ 200: (a) PNP as the standard; (b) hydroquinone as the standard; (c) sample drawn at 4 h growth interval; (d) sample drawn at 8 h growth interval; (e) sample drawn after overnight on PNP. The first peak with a retention time of 1.76/1.77 is the solvent peak.

type strain (Fig. 2); however, the smaller plasmid of app. 5 kb was absent from them. Such transconjugants were capable of utilizing PNP as sole source of carbon, nitrogen and energy and released nitrite ions from PNP as in the case of wild-type strain RKJ 200. These results, therefore, clearly demonstrate that the degradation of PNP is plasmid-encoded and that the genes for its degradation reside on a 50 kb plasmid.

The ability to resist the stress imposed by heavy metals is often ascribed to the extrachromosomal elements present in bacteria (29,31,32). Strain RKJ 200 and its PNP⁻ derivatives (spontaneous and mitomycin C derived) were tested for their resistance to different heavy metal salts which included zinc sulphate, sodium selenate, sodium selenite, cobaltous nitrate, sodium arsenite, sodium arsenate, mercuric chloride, potassium dichromate and ammonium molybdate. It was concluded that the gene(s) for zinc resistance (Zn^{++}) is encoded on the plasmid present in strain RKJ 200 since this strain was resistant to zinc sulphate whereas PNP⁻ derivatives were sensitive. Furthermore, the transconjugants (please see above) were also resistant to zinc sulphate. No other resistance marker(s) were identified as being carried by the plasmid(s) present in strain RKJ 200.

Although different reports provide some evidence for the presence of intermediates in the degradation of nitrophenols, the studies with the *Moraxella* sp. (30) and *Arthrobacter* sp. (15) are the only ones which detail the pathways for PNP degradation. In order to suggest the PNP degradative pathway in strain RKJ 200, TLC and GC studies were carried out. The TLC results are shown in Fig. 3. As shown in this figure, PNP and hydroquinone with Rf values of 0.78 and 0.64, respectively were detected in samples drawn at 4 h and 8 h intervals. The samples drawn after overnight growth did not show the presence of PNP or any other intermediates indicating the total degradation of PNP. To further confirm the presence of hydroquinone in the degradation pathway, GC studies were performed. The samples processed for TLC were
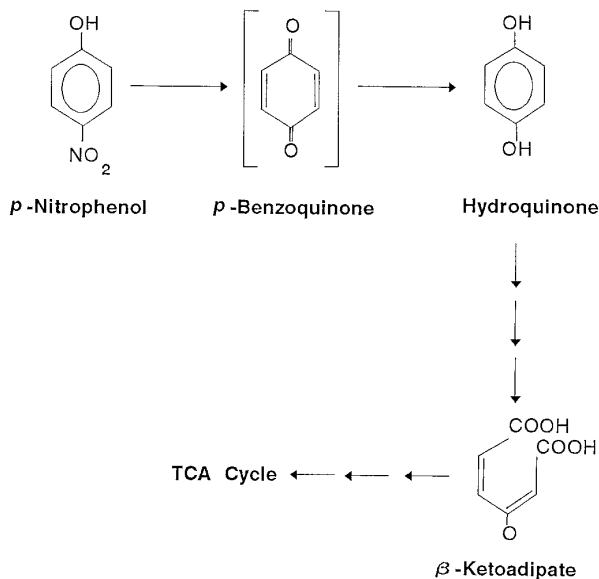
FIG. 5. Proposed pathway for the degradation of PNP by *P. cepacia* strain RKJ 200.

also analysed by gas chromatography (please see Materials and Methods). As shown in Fig. 4, presence of PNP and hydroquinone were present in sample drawn at 4 h interval based on the retention time of these compounds to that of the standards. This sample also showed the presence of possibly other intermediates with retention times of 3.092, 3.745 and 5.136, respectively (Fig. 4), which may be involved in the degradation of PNP. However, we did not make attempts to identify these compounds. The samples drawn after 8 h of growth showed the presence of only PNP whereas overnight grown sample did not show either PNP or hydroquinone. The TLC and GC studies, therefore, conclusively indicated the presence of hydroquinone during PNP degradation. Although we have not rigorously determined the further matabolism of hydroquinone in strain RKJ 200, it is likely that PNP is degraded via β-ketoadipate pathway. This is based on the observation that cells grown on PNP gave a positive Rothera test (13) with hydroquinone indicating the *Ortho* ring fission and subsequent formation of β-ketoadipate. Therefore, on the above basis, degradative pathway in strain RKJ 200 appears to be same as has been previously described by Spain and Gibson (30) in a *Moraxella* sp. An outline of this pathway is shown in Fig. 5.

The results presented above, on the basis of mutation, plasmid DNA isolation and conjugation studies, clearly demonstrate that the genes for the whole PNP degrading pathway are encoded on an app. 50 kb transmissible plasmid in a newly isolated *Pseudomonas cepacia* strain RKJ 200. To our knowledge, this is the first such conclusive and direct report. Furthermore, the gene(s) for resistance to heavy metal zinc is also located on the same plasmid. Therefore, such organism(s) could potentially prove useful not only for the decontamination of the pollutants but also for decontamination of the heavy metals in the environment. For a more detailed analysis of the regulation of synthesis of PNP degradative enzymes, we are presently cloning the genes for PNP degradation using broad host-range plasmid and cosmid vectors.

ACKNOWLEDGMENTS

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