Biochemical and Biophysical Research Communications **270,** 733–740 (2000)
doi:10.1006/bbrc.2000.2500, available online at http://www.idealibrary.com on IDEAL®

Plasmid-Encoded Degradation of *p*-Nitrophenol and 4-Nitrocatechol by *Arthrobacter protophormiae*

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Received February 29, 2000

Arthrobacter protophormiae **strain RKJ100 is capable of utilizing *p*-nitrophenol (PNP) as well as 4-nitrocatechol (NC) as the sole source of carbon, nitrogen and energy. The degradation of PNP and NC by this microorganism takes place through an oxidative route, as stoichiometry of nitrite molecules was observed when the strain was grown on PNP or NC as sole carbon and energy sources. The degradative pathways of PNP and NC were elucidated on the basis of enzyme assays and chemical characterization of the intermediates by TLC, GC, ^{1}H NMR, GC-MS, UV spectroscopy, and HPLC analyses. Our studies clearly indicate that the degradation of PNP proceeds with the formation of *p*-benzoquinone (BQ) and hydroquinone (HQ) and is further degraded via the β-ketoadipate pathway. Degradation of NC involved initial oxidation to generate 1,2,4-benzenetriol (BT) and 2-hydroxy-1,4-benzoquinone; the latter intermediate is then reductively dehydroxylated, forming BQ and HQ, and is further cleaved via β-ketoadipate to TCA intermediates. It is likely, therefore, that the same set of genes encode the further metabolism of HQ in PNP and NC degradation. A plasmid of approximately 65 kb was found to be responsible for harboring genes for PNP and NC degradation in this strain. This was based on the fact that PNP$^-$ NC$^-$ derivatives were devoid of the plasmid and had simultaneously lost their capability to grow at the expense of these nitroaromatic compounds.**

Key Words: **biodegradation; PNP pathway; 4-nitrocatechol pathway; plasmid-encoded;** ***Arthrobacter protophormiae.***

With the rapid thrust in industrial and agricultural activity, the recent times have witnessed vast quantities of soil and groundwater resources becoming contaminated with hazardous chemicals. Aromatic nitro compounds including nitrophenols, nitrobenzoates and nitrotoluenes are widely used in the manufacture of pesticides, dyes, explosives, drugs etc. (1–3). Nitrophenols can also accumulate in the soil as a result of hydrolysis of several organophosphorous insecticides, such as parathion or methylparathion, or from the use of other nitrophenolic herbicides (3–5).

Even though the nitro group enhances the resistance of the aromatic ring against biodegradation (2, 3) many bacteria have the ability to degrade nitrophenols (2, 3, 6–8). Aerobic biodegradation of PNP can be initiated by formation of either hydroquinone (HQ) or 4-nitrocatechol (NC) depending on the microorganism. *Moraxella* sp. and *Pseudomonas* sp. convert PNP to HQ (9–11), whereas *Arthrobacter* sp., *Flavobacterium* sp. and *Rhodococcus* sp. degrade PNP via the formation of NC (12–16). Another preliminary characterization of a novel monooxygenase from *Bacillus sphaericus* JS905 that catalyzed the first two steps in the degradation of PNP via NC and BT was recently reported (14). This alternate pathway of PNP involved the formation of NC which was oxidized to 2-hydroxy-1,4-benzoquinone (HBQ); this intermediate was then converted to BT which was cleaved to generate maleylacetate and β-ketoadipate, respectively.

Recently, our group reported a novel pathway for the oxidative degradation of NC by *Burkholderia cepacia* RKJ200 (17). The first step involved the conversion of NC into BT as described earlier (13). BT was then converted to HQ via the formation of HBQ and BQ that were further degraded via γ-hydroxymuconic semialdehyde and β-ketoadipate as intermediates; the unprecedented step in the NC pathway here was the reductive dehydroxylation of HBQ into BQ. Reductive dehydroxylation is a well known reaction in case of anaerobic metabolism of aromatic compounds by different denitrifying and phototrophic bacteria, but it was reported to be operative aerobically in *B. cepacia* RKJ200 for the first time (17).

The present communication reports the oxidative degradation of PNP via HQ formation which is not the

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0006-291X/00 $35.00

preferred route by gram-positive bacteria, and the NC degradation pathway which involves a similar reductive aerobic dehydroxylation of HBQ with the formation of BQ and HQ in *Arthrobacter protophormiae* RKJ100 similar to the earlier report in *B. cepacia* RKJ200 (17). Furthermore, the genes for the whole PNP and NC degradative pathways are encoded on the same plasmid.

MATERIALS AND METHODS

Bacterial strains, growth conditions and enzyme assays. *Arthrobacter protophormiae* strain RKJ100 and its cured derivatives have been described earlier (18). PNP, NC, BT and HQ were added as the filter sterilized solution to a final concentration of 0.3–0.5 mM after autoclaving the medium (9). Growth on the above compounds, preparation of crude and ultracentrifuged cell extracts, enzyme analyses and spectral analyses were performed as described previously (17, 19). PNP depletion (20), NC depletion (21) and nitrite release assay (22) were performed as described.

Respirometry, thin layer chromatography and gas chromatography studies. Oxygen uptake studies by whole cells were performed polarographically using Orion 860 oxygen meter as described earlier (17). Thin layer chromatography (TLC) using the precoated silica gel 60 F_{254} plates and gas chromatography (GC) studies using Hewlett-Packard 5890 series II GC were performed essentially in the same manner as described previously (9, 17).

Ultraviolet spectrophotometry, ^1H NMR, GC-MS and HPLC analysis. Ultraviolet spectrometry analysis for spectral analysis using cell free extracts was carried out as described earlier (17). Protein estimation was performed by the method of Lowry (23). ^1H NMR analyses of the samples carried out by blocking the ring cleavage using 2,2′-dipyridyl was performed as indicated previously (13, 17, 24). GC-MS analyses and HPLC analyses were also performed essentially as described (17).

Chemicals. PNP, NC, BT, (BQ), HQ and 2,2′-dipyridyl were purchased from Sigma Chemical Co. (U.S.A.). 2-Hydroxy-1,4-benzoquinone (HBQ) was prepared nonenzymatically using BT as substrate as shown earlier (25, 26). All other chemicals used were of the highest purity available.

RESULTS AND DISCUSSION

Growth on PNP and NC. The isolation of *Arthrobacter protophormiae* strain RKJ100 from pesticide contaminated agricultural land and its identification have been reported earlier (18). In liquid cultures the yellow color of PNP (0.5 mM final concentration) and NC (0.4 mM final concentration) changed to colorless, indicating their utilization by the microorganism. Microbial growth was measured by the increase in optical density (O.D.) at 600 nm. The microorganism released nitrite ions in the medium, suggesting the involvement of an initial oxidative step in the degradation of PNP and NC. When RKJ100 was grown in the presence of another nitrogen source (ammonium sulphate), stoichiometric amount of nitrite was released into the medium concomitant with the degradation of PNP (Fig. 1A) and NC (Fig. 1B).

Metabolic pathway for PNP degradation. Preliminary analysis of the catabolic pathway was performed by oxygen uptake studies. *Arthrobacter protophormiae* RKJ100 following growth on PNP showed enhanced oxygen uptake rates against PNP (0.49 μmol O_2 consumed min^{-1} mg^{-1} of protein) and HQ (0.44 μmol O_2 consumed min^{-1} mg^{-1} of protein) but not when BT was used as the substrate. Further, no oxygen uptake was observed when the cells were grown on glucose, indicating the inducible nature of the pathway enzymes. This indicated that PNP is degraded via the formation of HQ.

TLC and GC studies were carried out to further establish the pathway. TLC studies, following growth of RKJ100 on PNP, indicated the presence of HQ (R_f 0.65) and PNP (R_f 0.75) in samples drawn from 2 to 10 h intervals. However, the samples drawn after overnight growth did not show the presence of PNP or any other intermediates indicating the total degradation of PNP. Furthermore, when TLC plates were sprayed with Folin-Ciocalteu's reagent an immediate blue coloration was apparent in case of suspected HQ spots since *ortho* or *para* diphenols give an immediate blue coloration with this reagent (17, 27). When GC studies on the samples processed for TLC as indicated above were performed, presence of PNP (4.22 min) and HQ (2.55 min) was indicated based on their retention times in samples drawn from 2 to 10 h period. However, no PNP or HQ peaks were observed in overnight drawn samples, again suggesting the total degradation of PNP. We also carried out inhibition studies using 2,2′-dipyridyl since the presence of this compound in the growth medium chelates ferrous ions required for the ring-cleavage of aromatic compounds (11, 17, 21, 24). When RKJ100 was grown on PNP along with glucose as additional carbon source with 2,2′-dipyridyl and samples analyzed by TLC and GC, there was accumulation of HQ even after 48 h of growth, suggesting the inhibition of the ring cleavage of HQ.

GC and GC-MS studies were also performed using cell extracts of *Arthrobacter protophormiae* RKJ100 which were incubated with PNP (0.5 mM). After 3 h of incubation the metabolites were extracted and analyzed. GC studies indicated the appearance of an additional peak at 2.0 min in addition to PNP and HQ which corresponded well with standard BQ. When the above sample was analyzed by GC-MS studies, presence of BQ and HQ was again confirmed (please see Table 1 for mass spectral properties of BQ and HQ) in the pathway of PNP degradation. It was further indicated that HQ is degraded via β-ketoadipate pathway based on the fact that cells grown on PNP gave a positive Rothera test (28) with HQ suggesting the subsequent formation of β-ketoadipate. Taken together, the above results clearly indicated that the degradation of PNP in RKJ100 occurs via the same pathway as reported earlier by Spain and Gibson (11) in a *Moraxella* sp. and by Prakash *et al.* (9) in *Burkholderia cepa-*

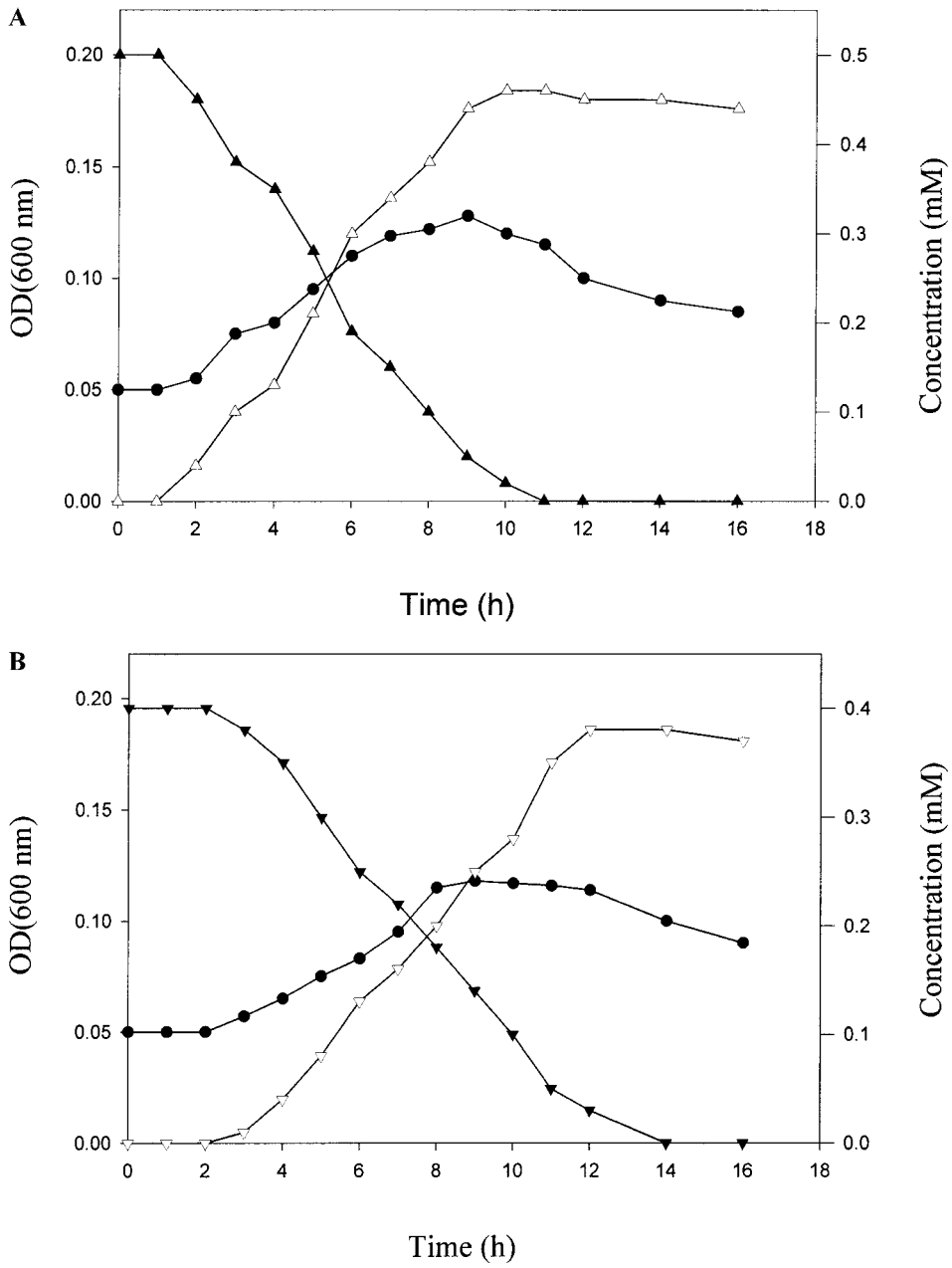

FIG. 1. (A) Degradation of PNP by *Arthrobacter protophormiae* RKJ100. PNP is degraded with concomitant release of nitrite molecules. The inoculum used was obtained from washed cells of RKJ100 grown overnight on PNP. ●, The optical density (OD) of culture; ▲, PNP depletion; △, nitrite released in the medium. (B) Degradation of NC by *Arthrobacter protophormiae* RKJ100. NC is degraded with concomitant release of nitrite molecules. The inoculum used was obtained from washed cells of RKJ100 grown overnight on NC. ●, The optical density (OD) of culture; ▼, NC depletion; ▽, nitrite released in the medium.

cia RKJ200 (earlier known as *Pseudomonas cepacia* RKJ200). This pathway is shown in Fig. 2.

Metabolic pathway for NC degradation. Oxygen uptake studies were carried out initially to have an indication of the NC degradative pathway metabolites. *Arthrobacter protophormiae* strain RKJ100 after growth on NC exhibited enhanced oxygen uptake rates against NC (0.46 μmol O_2 consumed min^{-1} mg^{-1} protein), BT (0.48 μmol O_2 consumed min^{-1} mg^{-1} protein) and HQ (0.42 μmol O_2 consumed min^{-1} mg^{-1} protein), respectively. No oxygen uptake was observed when the cells were grown on glucose, indicating the inducible nature of the pathway enzymes. This indicated that NC is degraded via BT and HQ formation.

In order to further elucidate the NC degradative pathway TLC and GC studies were undertaken. TLC studies, following growth of RKJ100 on NC, indicated the presence of HQ (R_f 0.66) and NC (R_f 0.55) in sam-

TABLE 1

GC-MS and ^{1}H NMR Characterization of the Metabolites Formed from the Degradation of NC by *Arthrobacter protophormiae* RKJ100

Retention time (min) (GC-MS)	Mass spectral properties {*m/z* (% relative intensity) [molecular ion]}	^{1}H NMR chemical shifts (δ) and coupling constants (*J* values in Hertz)	Metabolite identified as[a]
1.90	108(51)[M^+], 82(23), 80(22), 54*(100)	ND[b]	*p*-Benzoquinone (BQ)
5.00	110*(100)[M^+], 82(16), 81(30), 55(33), 54(27), 53(42)	δ: 6.64 (4 H, s, H-2, H-3, H-5 and H-6)	Hydroquinone (HQ)
9.17	126*(100)[M^+], 97(15), 80(37), 69(13), 55(22), 53(32), 52(98), 51(32), 42(23), 41(23)	δ: 6.12 (1 H, dd, J = 2.8 Hz, 8.5 Hz, H5); 6.35 (1 H, d, J = 2.8 Hz, H-3); 6.58 (1 H, d, J = 8.5 Hz, H-6)	1,2,4-Benzenetriol (BT)
13.30	155*(100)[M^+], 139(5), 125(31), 109(25), 107(21), 97(10), 81(51), 79(23), 63(32), 55(60), 53(87), 51(66)	δ: 6.90 (1 H, d, J = 8.8 Hz, H-6); 7.63 (1 H, dd, J = 2.8 Hz, 8.8 Hz, H-5); 7.70 (1 H, d, J = 2.8 Hz, H-3)	4-Nitrocatechol (NC)

[a] The metabolites were identified after comparison with standard compounds.
[b] ND, not detected; BQ is not detectable in whole cell studies.

ples drawn from 2 h to 10 h intervals. However, the samples drawn after overnight growth did not show the presence of NC or any other intermediates indicating the total degradation of NC; when TLC plates were sprayed with Folin-Ciocalteu's reagent an immediate blue coloration was evident in case of suspected HQ spots since *ortho* or *para* diphenols give an immediate blue coloration with this reagent (17, 27). GC studies on the samples processed for TLC above were also performed. The presence of NC (7.55 min) and HQ (2.58 min) was indicated by their retention time in samples drawn at 2 to 10 h time intervals. However, no peaks were observed in overnight drawn samples, again suggesting the total degradation of NC. Another peak with the retention time of 3.75 min could correspond well with that of standard BT in the above samples. When inhibition studies using 2,2′-dipyridyl were carried out, accumulation of BT and HQ could be seen in GC studies. We have earlier shown that, in a *Burkholderia cepacia* RKJ200, NC is degraded via BT and HBQ formation which is reductively dehydroxylated to BQ and HQ and ring cleavage takes place thereafter (17). To check whether a similar kind of reaction is taking place in the degradation of NC by *Arthrobacter protophormiae* RKJ100, we performed experiments with cell extracts induced with NC since we were unable to detect HBQ and/or BQ by TLC and GC analyses using whole cells. The extracts were incubated with BT indicative of the formation of HBQ (13, 17). The samples were then analyzed by GC after 3 h of incubation to check the degradation products which showed the presence of BQ (2.0 min) in addition to BT (3.78 min) and HQ (2.58 min) suggesting the conversion of BT to HQ via BQ. Presence of BQ was also checked by co-elution studies. As reported earlier (17), we also confirmed that the conversion of BT (λ_{max} 286 nm) into HBQ (λ_{max} 260 nm) using cell extracts of RKJ100 was enzymatic since nonenzymatic conversion of BT into HBQ was found to be much more slow based on UV spectral scanning experiments (data not shown). This clearly showed that conversion of BT into BQ via HBQ involves enzymatic reaction(s).

UV spectral analysis with cell extracts of RKJ100 was also carried out by incubating them with HBQ, BQ and HQ separately. The analysis of products formed based on the absorption maxima from these substrates indicated that BQ (λ_{max} 240 nm) is formed from HBQ (λ_{max} 260 nm) and HQ (λ_{max} 289 nm) from BQ (λ_{max} 240 nm). γ-Hydroxymuconic semialdehyde (λ_{max} 292 nm) was formed from HQ (λ_{max} 289 nm) indicating its ring fission. The presence of HBQ, BQ and HQ was also confirmed by co-scanning studies using authentic compounds. Extracts from cells grown on NC gave a positive Rothera test (28) with NC, BT, HBQ, BQ and HQ indicating subsequent formation of β-ketoadipate in the degradative pathway.

Further identification of the metabolites in NC degradative pathway came from ^{1}H NMR and GC-MS analyses. The presence of NC, BT and HQ in the extracted samples using whole cells was confirmed by the analysis of its ^{1}H NMR data (Table 1) which showed the characteristic peaks corresponding well with the ^{1}H NMR of the authentic compounds. The GC-MS analysis using cell extracts showed BQ (1.90 min), HQ (5.00 min) and BT (9.17 min) as determined by comparing their mass spectral properties (Table 1) with that of authentic compounds. The key intermediate 2-hydroxy-1,4-benzoquinone (HBQ) with the retention time of 2.40 min and molecular ion [M^+] at m/z 124 corresponding to the mass of HBQ and major fragmented ions at m/z 108 ($M^+ - O$), 96 ($M^+ - CO$) and 79 ($M^+ - COOH$) (Fig. 3) was identical to the mass spectral properties of the authentically prepared HBQ as also reported recently by our group (17).

For quantitation studies, HPLC analyses was carried out to determine the stoichiometry and rate of conversion of NC into BT and HQ, and BT into HQ (17). The concentrated cell suspensions were incubated with

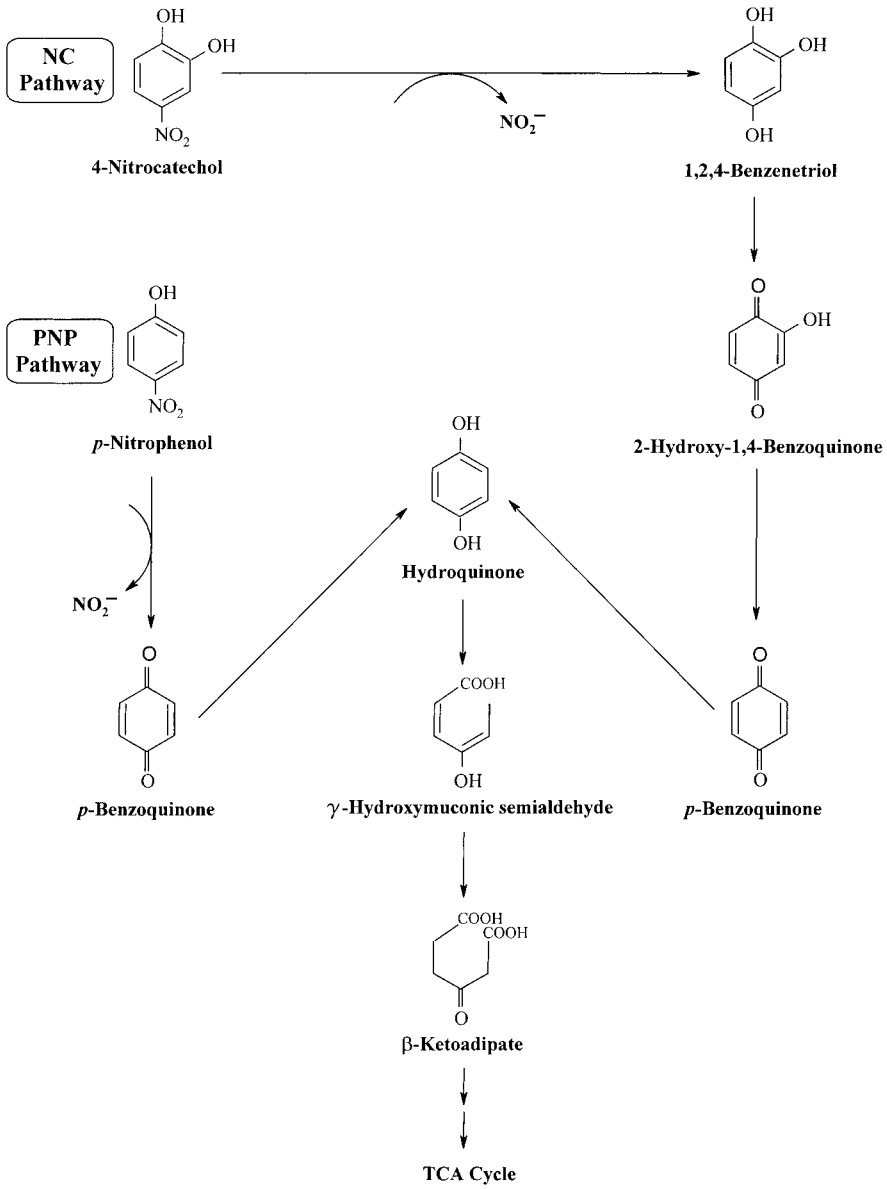

FIG. 2. Proposed pathways for the degradation of PNP and NC by *Arthrobacter protophormiae* strain RKJ100.

NC (0.4 mM), and BT (0.4 mM) separately in presence and absence of 2,2′-dipyridyl. In the presence of 2,2′-dipyridyl, three compounds were detected after 2 h of incubation: 0.35 mM NC (12.8 min), 0.03 mM BT (3.9 min) and 0.02 mM HQ (4.7 min); after 4 h NC depleted (0.2 mM) giving rise to 0.08 mM of BT and 0.06 mM of HQ. After 6 h, 0.13 mM of NC was detected with BT (0.1 mM) and HQ (0.15 mM) accumulating in the medium. These results are shown in Fig. 4. When the same studies were carried out without 2,2′-dipyridyl, after 4 h, only 0.15 mM of NC was detected forming BT (0.1 mM) and HQ (0.1 mM) and, after 6 h, there was complete utilization of NC with traces of BT (0.02 mM) and HQ (0.2 mM) was the only prominent compound present in the medium (Fig. 4). There was complete utilization of all these compounds after 10 h of incubation. Similarly when concentrated cell suspensions were incubated with BT and 2,2′-dipyridyl was incorporated in the growth medium, after 2 h there was depletion of BT (0.35 mM detected) and appearance of HQ (0.05 mM). After 4 h, 0.25 mM of BT was detected giving rise to HQ (0.1 mM) and after 6 h of incubation there was very minute BT (0.05 mM) and HQ accumulated (0.3 mM) in the medium. In the absence of 2,2′-dipyridyl, BT depleted after 4 h (0.1 mM remaining) forming HQ (0.15 mM). After 6 h BT was completely utilized forming HQ (0.1 mM) which was also completely utilized after 8 h generating lower pathway intermediates. These results are shown in Fig. 5. It may be mentioned here that attempts to quantitate

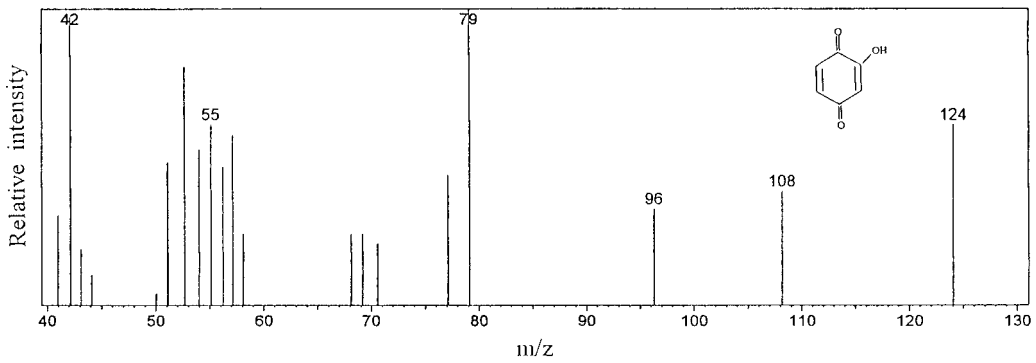

FIG. 3. Mass spectrum of HBQ isolated from cell extracts of *Arthrobacter protophormiae* strain RKJ100 following incubation with NC.

HBQ and BQ by HPLC studies were not made since these compounds remain undetectable in whole cell studies (17). The instability and highly reactive nature of these intermediates (6, 17, 25) may be the reason for their efficient uptake inside the cell as soon as they are formed in order to prevent their unfavorable coupling reactions which would hinder with their further degradation.

On the basis of the studies carried out by enzyme assays, TLC, GC, ^{1}H NMR, GC-MS, UV spectrophotometry and quantitation of metabolites by HPLC described above the pathway for the degradation of NC has been shown in Fig. 2; this pathway is similar as reported earlier in case of *B. cepacia* RKJ200 (17).

Location of genes for PNP and NC degradation. We have reported the presence of an approximately 65 kb plasmid in *Arthrobacter protophormiae* strain RKJ100 which encodes the genes for a novel ONB degradative pathway (18). **A**ttempts were made to determine if the same plasmid has an involvement in PNP and NC degradation as well. The mitomycin C derived cured ONB$^-$ derivatives reported earlier (18) were also found to be incapable of utilizing PNP and NC as sole source of carbon and energy. Thus these PNP$^-$ NC$^-$ derivatives were analyzed to confirm the same. When two such derivatives were analyzed by TLC, GC and spectral analysis it showed that no degradation of PNP or NC occurred except for the non-enzymatic conversion of BT into HBQ (spectral analysis; data not shown). The above results demonstrate that the genes encoding PNP and NC degradation also reside on the same plasmid which encodes genes for ONB degradation in RKJ100.

Since HQ is an intermediate in PNP as well as NC degradation, it is likely that the same set of genes

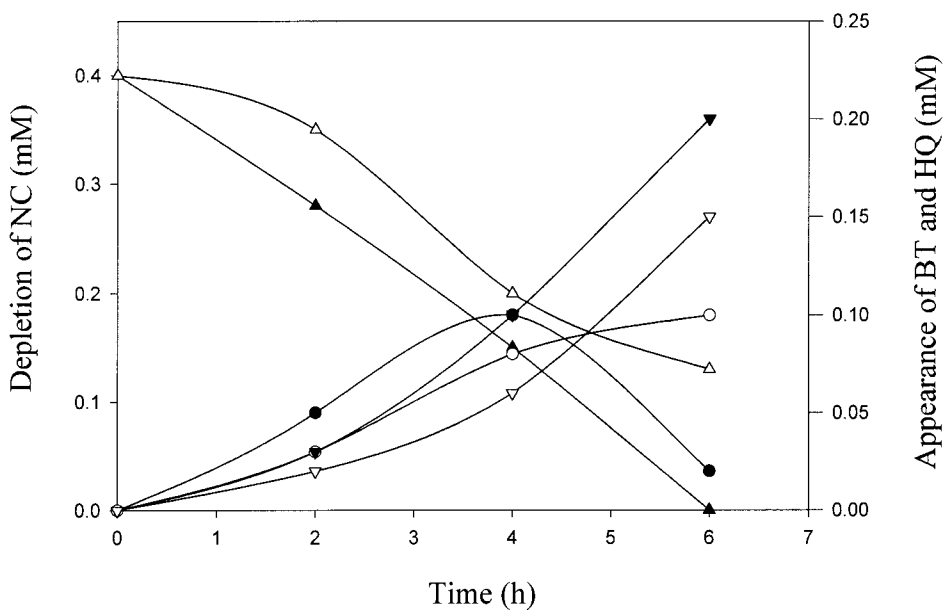

FIG. 4. Quantitative conversion of NC into BT and HQ by *Arthrobacter protophormiae* strain RKJ100. NC depletion (△) and appearance of BT (○) and HQ (▽) when the ring cleavage was blocked with 2,2′-dipyridyl; NC depletion (▲) and appearance of BT (●) and HQ (▼) in the absence of 2,2′-dipyridyl.

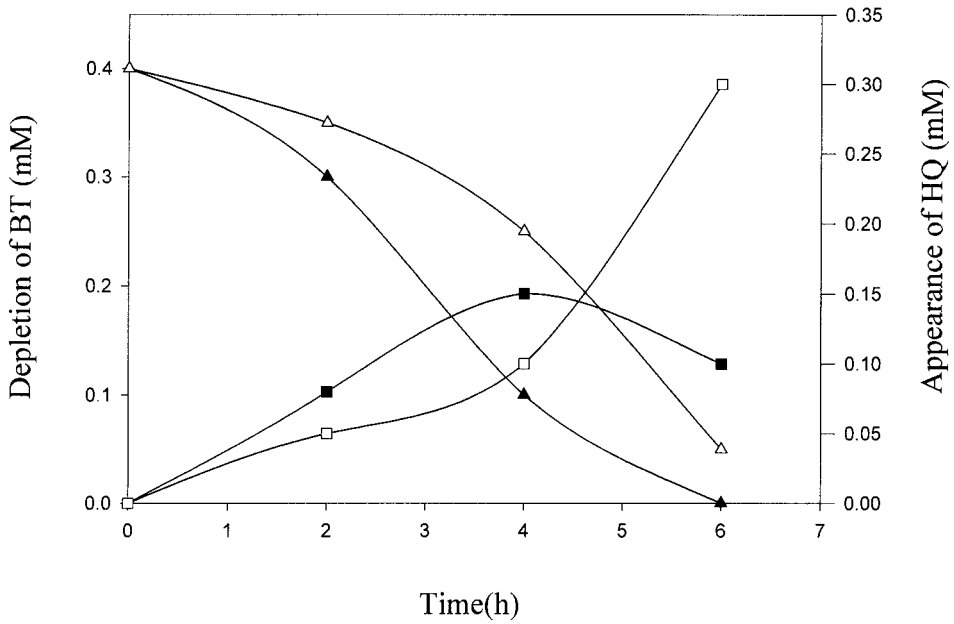

FIG. 5. Quantitative conversion of BT into HQ by *Arthrobacter protophormiae* strain RKJ100. BT (△) depletion and appearance of HQ (□) when the ring cleavage was blocked with 2,2′-dipyridyl; BT depletion (▲) and appearance of HQ (■) in the absence of 2,2′-dipyridyl.

encode the further metabolism of HQ via the β-ketoadipate pathway (Fig. 2). We are presently cloning the genes for PNP and NC degradation to understand the molecular regulation of the degradation and to establish whether conversion of PNP to HQ and NC to HQ are contiguous or not. Also, since the pathways of PNP and NC are similar in *Arthrobacter protophormiae* RKJ100 and *Burkholderia cepacia* RKJ200 (9, 17), it would be very interesting to compare genes of such strains to assess whether they share a common origin or arose independently. Furthermore, this is the first report in which a soil isolate is reported to degrade PNP and NC oxidatively and ONB reductively by oxygen-insensitive reductase(s) which are encoded on the same plasmid (18). The bioremediation potential of such a microorganism is worthy of attention since it could prove to be very efficient in the clean-up of sites contaminated with different nitroaromatic compounds.

ACKNOWLEDGMENTS

This work was supported by the Council of Scientific and Industrial Research and Department of Biotechnology, India. We are thankful to Mr. Avtar Singh, RSIC, Panjab University, Chandigarh for his help in carrying out the ^{1}H NMR analyses and to Mr. Sanjeev Sharma, Ms. Kamaljeet Kaur and Mr. Vikas Grover, NIPER, SAS Nagar, for carrying out the GC-MS and HPLC analyses. A.C. acknowledges a Senior Research Fellowship by Council of Scientific and Industrial Research, India. This is IMTECH Communication No. 003/2000.

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