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ORIGINAL ARTICLE

Isolation and characterization of a bacterial strain of the genus *Ochrobactrum* with methyl parathion mineralizing activity

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Abstract

Aims: To isolate and characterize a methyl parathion (MP)-mineralizing bacterium, and to elucidate the degradative pathway of MP and localize the responsible degrading genes.

Methods and Results: A bacterial strain, designated B2, capable of mineralizing MP was isolated from the MP-polluted soil. Analysis of the 16S rRNA gene sequence and phenotypic analysis suggested that strain B2 had a close relationship with *Ochrobactrum anthropi*. B2 could totally degrade MP and four metabolites [*p*-nitrophenol (PNP), 4-nitrocatechol (4-NC), 1,2,4-benzenetriol (BT) and hydroquinone (HQ)] were identified by HPLC and gas chromatography-mass spectrometry analyses. Plasmid curing of strain B2 resulted in the loss of ability of B2 to degrade PNP, but not the ability to hydrolyse MP.

Conclusions: *Ochrobactrum* sp. B2 can mineralize MP rapidly via PNP, 4-NC, BT and HQ pathway. B2 harbours a plasmid encoding the ability to degrade PNP, while MP-hydrolysing activity is encoded on the bacterial chromosome.

Significance and Impact of the Study: This new bacterial strain (B2) capable of mineralizing MP will be useful in a pure-culture remediation process of organophosphate pesticides and their metabolites such as nitroaromatics.

Introduction

The continuous use of organophosphorus insecticides in large quantities for the control of a wide range of insects throughout the world and their potential neurotoxicity to the nontarget organisms including humans have raised serious concern and have led to the development of safe strategies to deal with its widespread dispersal in the ecosystem (Chapalamadugu and Chaudhry 1992). Microbial degradation is considered to be a major factor determining the fate of organophosphorus insecticides in the environment. Studies of microbial degradation are useful in the development of strategies for the detoxification of the insecticides by micro-organisms.

Methyl parathion (*O*,*O*-dimethyl-*O*-*p*-nitro-phenyl-phosphorothioate, MP) is an extremely toxic compound which is still commonly used in some countries. Although some studies on microbial degradation of MP have been conducted (Chaudhry *et al.* 1988; Ou and Sharma 1989; Cui *et al.* 2001; Chen *et al.* 2002), reports on microbes that grow on MP as sole source of both carbon and phosphorus appear to be rare (Ramanathan and Lalithakumari 1999; Chen *et al.* 2002; Liu *et al.* 2005). In this report, we present results on the isolation and characterization of a bacterial strain *Ochrobactrum* sp. B2, which shows significant ability of degrading MP and its intermediates. In addition, we propose a pathway for the complete breakdown of MP with the release of nitrite as a catabolic by-product.

Material and methods

Chemicals

Methyl parathion (99·1%) was purchased from the Institute for the Control of Agrochemicals, Ministry of Agriculture of China, Beijing, China. 4-Nitrocatechol (98%, 4-NC) was purchased from Fluka (Bucks, Switzerland); 1,2,4-benzenetriol (99%, BT) from Aldrich (Milwaukee, WI, USA); hydroquinone (>98%, HQ) from Tianjin Chemical Plant (Tianjin, China); 2,2′-dipyridyl (>99·9%) from Beijing Siying Chemical Plant (Beijing, China). All other chemicals used were of the highest purity available.

Isolation of an MP-degrading bacterium by enrichment culture

Enrichment was performed by successive sub-culturing of samples. One gram of soil sample, obtained from the yard of a pesticide factory (located in Gaomi, Shandong Province, China) which manufactures organophosphorus insecticides (OPs), was used as the inoculum for 50 ml soil enrichment medium (peptone 10 g, glucose 1 g, $KH_2PO_4$1 g, NaCl 1 g in 1 l sterile water, pH 7·2, Li *et al.* 1996) supplemented with MP (50 mg l^{-1} final concentration). Such enriched cultures were incubated at 28°C with shaking (150 rev min^{-1}) for a week. Enrichment culture (5 ml) was subcultured into 50 ml fresh enrichment medium containing MP gradually from 50 to 500 mg l^{-1} for nine generations. The enriched cultures were then transferred into minimal salt medium (MSM) (Sutherland *et al.* 2000) supplemented with 50 mg l^{-1} MP as the sole carbon source. After subculturing in MSM with increasing concentration of MP up to 300 mg l^{-1} for six generations, pure cultures were obtained by performing appropriate serial dilutions of the enrichment culture in MSM medium and plating them onto MSM plates containing 100 mg l^{-1} MP. Fifty colonies were picked randomly, re-streaked to ensure purity. The colonies were inoculated into MSM medium containing 100 mg l^{-1} MP. Isolates that grew fast and quickly turned the culture yellow were selected for further investigation. The yellow colour is indicative of *p*-nitrophenol (PNP), which is a product of MP hydrolysis.

Identification of micro-organism

An isolate (CGMCC No. 1221), designated B2, capable of degrading MP was further characterized morphologically, biochemically and was deposited in China General Microbiological Culture Collection Center. The 16S rRNA gene of B2 was amplified by PCR with a set of primers (27f: 5′-AGA GTT TGA TCC TGG CTC AG-3′ and 1492r: 5′-TAC GGT TAC CTT GTT ACG ACT T-3′) (Weisburg *et al.* 1991). The PCR product of 16S rRNA was sequenced by Bioasia Company (Shanghai, China). Identification was achieved by comparing the sequence data obtained with 16S rRNA sequence data from type strains available at the public database of GenBank (http://www.ncbi.nlm.nih.gov/) using the BlastN program. The 16S rRNA gene sequence has been deposited in the GenBank Database under accession no. AY661464.

MP biodegradation studies

The inocula for all the experiments of MP degradation studies were prepared by growing bacteria in 50 ml of MSM supplemented with 0·1% glucose and 200 mg l^{-1} MP and incubated for 36 h at 30°C on a shaker at 150 rev min^{-1}. The culture was asceptically harvested and the cells washed thoroughly with MSM (8000 ***g***, 10 min, 4°C). The cells were suspended in sterile MSM to an OD_{600} of 1·0. To 4·9 ml of MSM amended with 100 mg l^{-1} MP was added 0·1 ml of inoculum and incubated on a rotary shaker (150 rev min^{-1}) at 30°C. After regular intervals, samples were taken for quantitation of cell growth, MP, PNP, nitrite ion, and metabolite identification. All the experiments were performed in triplicate. Un-inoculated control tubes were maintained in all experiments.

Portions of the cultures were extracted twice with equal volumes of ethyl acetate, and extracts were dehydrated with sodium sulfate. These extracts were filtered (0·45 μm) and used to determine MP degradation and identify metabolites. Samples of 1 μl (diluted if necessary) were analysed using a Hewlett-Packard 5890 II GC (Hewlett-Packard, Wilmington, DE, USA) equipped with ECD detector and a capillary HP-1 column (polydimethylsiloxane 25 m × 0·32 mm × 0·25 μm) with the uninoculated culture as a control. The detector, injector and column temperatures were 300, 300 and 200°C, respectively. Column pressure was 7·7 psi, column flow was 0·932 ml min^{-1}. Under these conditions, the retention time of MP was 8 min. MP concentration was determined by comparing peak area of the samples to a standard curve. Gas chromatography-mass spectrometry (GC-MS) was obtained on an Agilent Technologies 6890N GC-5973N MSD (Agilent Technologies, Palo Alto, CA, USA). The GC was equipped with a DB-5MS (95% polydimethyl siloxae 5% poly-1,4-bis-dimethylsiloxae phenylene siloxae, 60 m × 0·25 mm ID, film thickness 0·25 μm) capillary column and operated in splitless mode. Helium (>99·999%) was used as carrier gas with

a constant flow rate of 1 ml min^{-1}. The inject temperature was 250°C and the GC-MS transfer line temperature was 280°C, ion source 230°C, quadrupole 150°C. All samples were analyzed with 70 eV nominal electron energy and scan range 30–300 amu.

Portions of the supernatants of cultures (10 000 ***g***, 30 min, 4°C) were filtered (0·22 μm). The pelleted cells were washed thoroughly with sterile distilled water (8000 ***g***, 10 min, 4°C) and suspended in sterile water. The growth of cells was determined by monitoring the OD_{600} of suspended cells. The filtrates were used to determine the concentrations of PNP and nitrite ion, and to identify the metabolites. Nitrite ion was quantitatively determined based on the standard curves prepared using sodium nitrite (DU-800 spectrophotometer; Beckman Coulter, Fullerton, CA, USA) by the method of Montgomery and Dymock (1961). PNP was quantified by HPLC (Agilent 1100). HPLC studies were conducted at room temperature using Agilent Zorbax 300SB-C18 column and acetonitrile : water = 15 : 85 (water contains acetic acid, 650 : 1, v/v; pH = 3·0) as the mobile phase at a flow rate of 0·700 ml min^{-1}. The analysis was performed at 290 nm (DAD detector), column pressure of 71 bar, column temperature at 40°C. Sample volume was 5 μl.

Quantitative analysis of intermediate in PNP degradation by B2

p-Nitrophenol degradation study and quantitative analysis of intermediates were performed by HPLC studies. B2 was harvested after growth on MSM supplemented with 50 mg l^{-1} of PNP for 36 h, and the washed concentrated cell suspensions were incubated with PNP at an initial OD_{600} of 0·03. 2,2′-dipyridyl (1 mmol l^{-1}) (inhibitor of HQ degradation) was also added in order to detect accumulating intermediates from PNP. Compounds were identified and by comparison of retention times with those of standards. Nitrocatechol, BT, and HQ concentrations were also calculated from their concentration curves respectively.

Plasmid curing and isolation

The plasmid curing was performed as described by Trevors (1986). In brief, a portion of overnight B2 culture in LB (Sambrook and Russell 2001) was subcultured twice into LB medium supplemented with 30 μg ml^{-1} acridine orange and grown at 40°C for 2 days. The culture was appropriately diluted and spread on to LB plates. Single colonies were checked for MP or PNP degrading activities. The plasmid of native and cured strains was isolated by the alkaline lysis method and then electrophoresed on a 0·8% agarose gel (Sambrook and Russell 2001).

Results

Isolation and characterization of an MP-degrading microbe

Thirteen different isolates using MP as the sole source of carbon were obtained from the MP-contaminated soil. Of these, one isolate, B2, showed good growth and high activity of MP hydrolysis and was chosen for further studies. The optimal growth conditions for this strain were pH 8–9 at 30°C (data not shown). Isolate B2 was found to grow in the medium supplemented with ampicillin up to 200 μg ml^{-1}, kanamycin to 150 μg ml^{-1}, streptomycin to 50 μg ml^{-1}, chloramphenicol to 10 μg ml^{-1}, while no growth was observed with rifampicin at a concentrate as low as 10 μg ml^{-1}.

The isolated strain B2 was rod shaped, Gram negative, oxidase and catalase positive. Full details of the biochemical and physiological characteristics of strain B2 are given in Table 1.

The 16S rRNA gene of isolate B2 was very similar in sequence to the *Ochrobactrum* strains. The highest degree of identity (100%) was obtained with two 16S rRNA gene sequences belonging to *Ochrobactrum* (AJ276036.1 and AF229875.1). Ninety-nine per cent of identity was found with *Ochrobactrum* sp. mp-5 (AY331579.1) and *Ochrobactrum* sp. mp-6 (AY331580.1). Analysis of the 16S rRNA gene sequence suggested that this strain B2 was closely related to *Ochrobactrum anthropi*. It was tentatively identified as an *Ochrobactrum* sp.

MP degradation

Degradation studies showed a rapid decrease in MP concentration, and prompt formation of a major metabolite, PNP (Fig. 1). MP was degraded quickly by B2: 40%

Table 1 Characteristics of strain B2

Characteristics	Strain B2	Characteristics	Strain B2
Morphology	Rod	Substrate utilization	
Gram-reaction	–	Glucose	+
Aerobic growth	+	Trehalose	+
Growth at 41°C	–	Rhamnose	+
Growth at 4°C	–	Xylose	+
Oxidase	+	Sucrose	+
Catalase	+	Fucose	+
Lipase (Tween 80)	–	Arabinose	+
Nitrate reduction	+	Mannose	+
Hydrolysis of gelatin	–	Galactose	+
Hydrolysis of esculin	–	Lactose	+
Arginine dihydrolase	–	Mannitol	+
Fluorescein	–	Sorbitol	–

+, positive; –, negative.

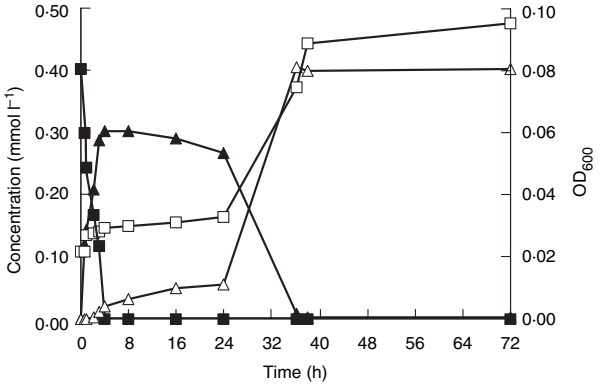

Figure 1 Growth of *Ochrobactrum* sp. B2 in minimal salt media containing methyl parathion (MP) and changes in the concentrations of MP, *p*-nitrophenol and nitrite ion. (■) MP, (▲) *p*-nitrophenol, (△) nitrite ion, and (□) OD_{600}.

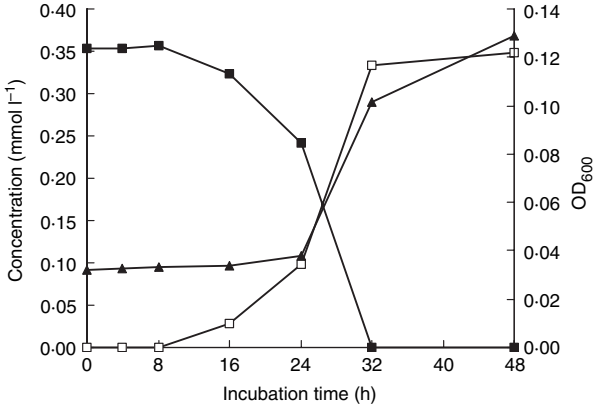

Figure 2 Growth of *Ochrobactrum* sp. B2 in minimal salt media containing *p*-nitrophenol. (■) *p*-nitrophenol, (□) nitrite ion, and (▲) OD_{600}.

decrease of MP content in the medium within the first hour. The concentration of MP decreased from 100 mg l^{-1} (0·4 mmol l^{-1}) to an undetected level in 4 h. PNP was observed within 0·5 h and it reached a peak in 4 h (Fig. 1). PNP remained at a relatively constant concentration for a period of about 20 h and decreased sharply afterwards. PNP degradation was almost completed (about 1% of maximal PNP produced during growth) in 38 h. No degradation of MP was observed in un-inoculated controls (data not shown).

B2 could grow on PNP, the intermediate of MP degradation (Figs 1 and 2). B2 growth was concomitant with the decrease of PNP and nitrite was released in stoichiometric quantities, which indicated that B2 could use PNP as carbon and energy sources. The growth of cells occurred after a significant lag, suggesting that the PNP degrading enzymes seem to be induced or are activated by the accumulation of nitrophenol.

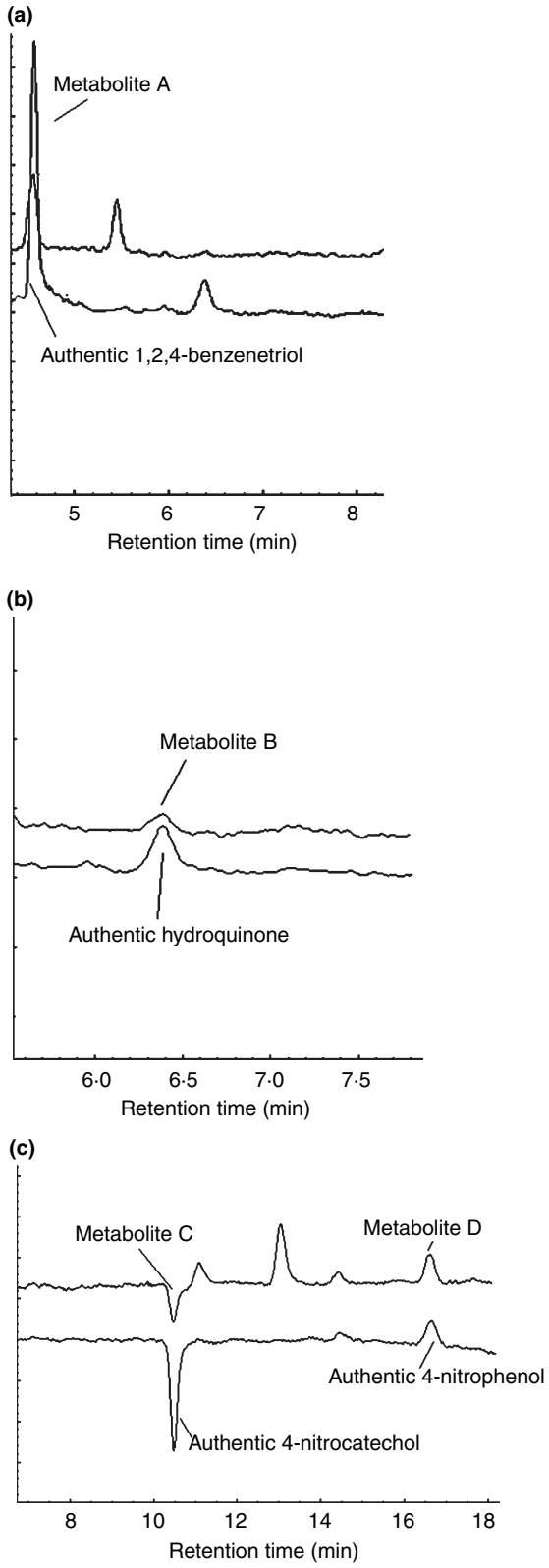

Figure 3 HPLC chromatogram of metabolites of methyl parathion degradation and authentic substances.

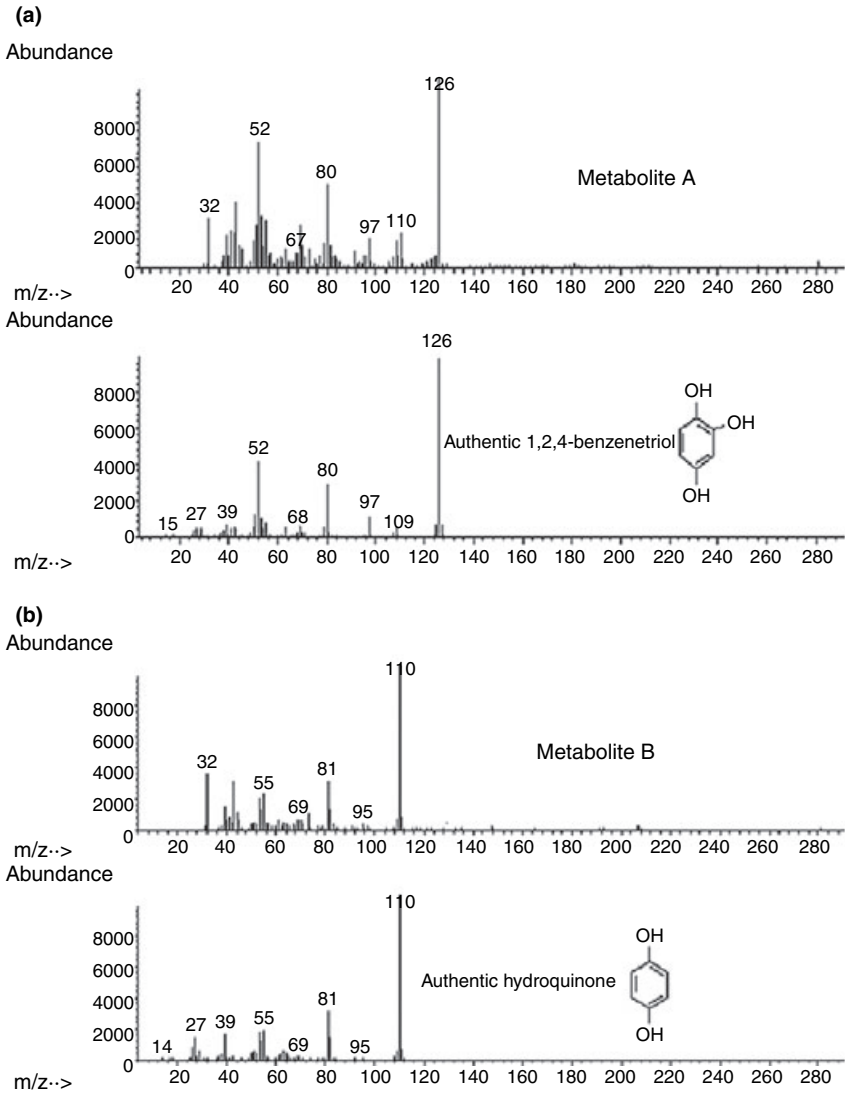

Figure 4 GC-MS spectra and structures of four intermediates of methyl parathion degradation. Figure 4 continued in next page.

Identification of metabolites

HPLC and GC-MS studies were performed using culture extracts of B2 that had been incubated with MP. HPLC studies revealed the appearance of additional peaks in the samples drawn from 30–38 h intervals not seen in the control samples, of which the retention times correlated well with those of authentic PNP, BT, HQ and 4-NC (Fig. 3). GC-MS analysis indicated the presence of four metabolites, identified as PNP, 4-NC, BT and HQ based on retention time and mass spectrum (Fig. 4a–d). Taken together, the above results indicated that PNP, 4-NC, BT and HQ were possible metabolites. GC and HPLC studies revealed that samples drawn after 72 h of growth did not show the presence of PNP or any other intermediates indicating total degradation of MP (data not shown).

To determine kinetics of appearance of intermediates in the pathway of PNP degradation, we quantified 4-NC, BT and HQ present in the medium during the growth period. Pre-experiments had shown that the process of degradation of the intermediates was fast upon the onset of B2 growth on PNP. Thus, 2,2′-dipyridyl, an inhibitor of the ring cleavage of some aromatic compounds such as HQ, was added during incubation. In the presence of 2,2′dipyridyl, two compounds were detected after 30 h of incubation: 48·75 mg l^{-1} PNP and 1·58 mg l^{-1} 4-NC; after 32 h, four compounds were detected: 39·39 mg l^{-1} PNP, 8·56 mg l^{-1} 4-NC, 0·85 mg l^{-1} BT and 1·58 mg l^{-1}HQ; after 34 h, PNP depleted to an undetected level, giving rise to 0·52 mg l^{-1} of 4-NC, 0·01 mg l^{-1} of BT, and the only prominent compound HQ (25·16 mg l^{-1}); after 36 h, only HQ (18·36 mg l^{-1}) was detected; no compound was found after 38 h of incubation (Fig. 5). When the same studies were

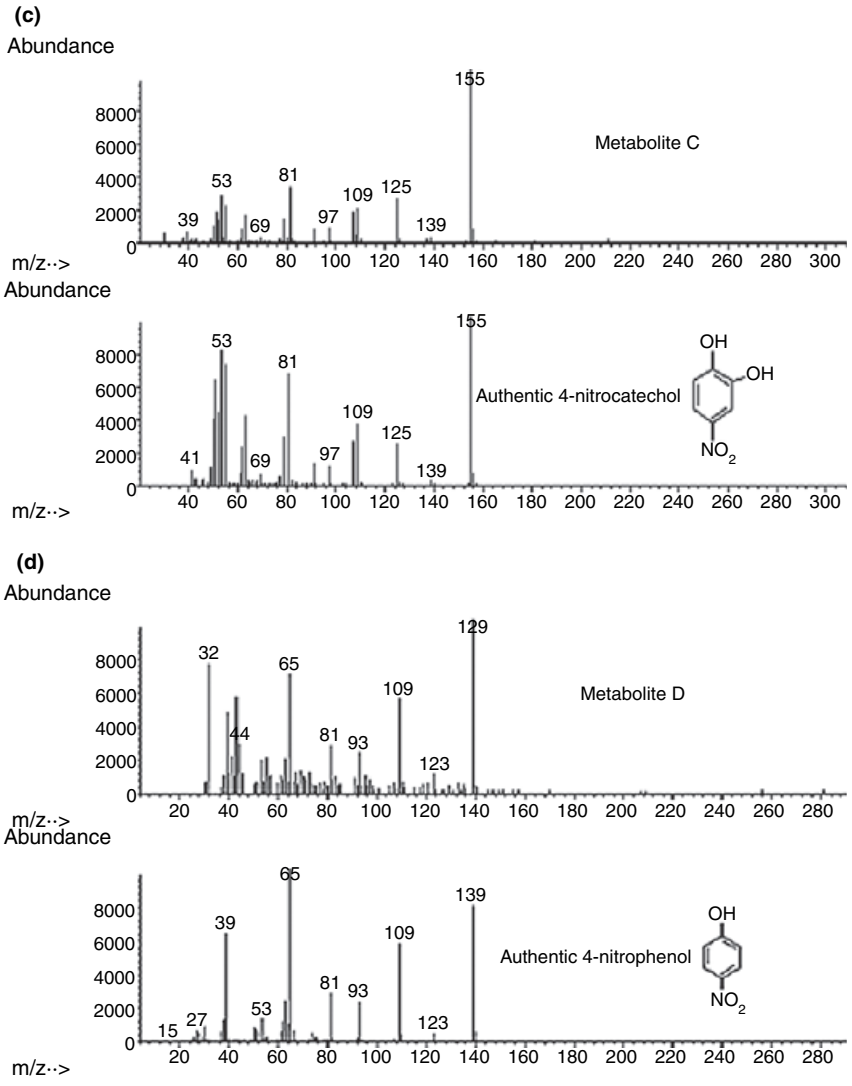

Figure 4 Continued

carried out without 2,2′-dipyridyl, there was complete utilization of PNP with traces of 4-NC (0·4 mg l^{-1}) and HQ (0·69 mg l^{-1}) after 28 h; after 30 h, only 0·04 mg l^{-1} of 4-NC and 1·15 mg l^{-1} of HQ were detected in the medium. There was complete utilization of all these compounds after 32 h of incubation.

Location of genes for MP and PNP degradation

A plasmid was detected in B2 (Fig. 6). To determine whether MP-degrading activity was controlled by a plasmid, we attempted to correlate loss of MP hydrolase activity with plasmid removal. Curing studies were conducted by growing B2 in a medium with 30 μg l^{-1} acridine orange at 40°C. Hundred colonies were picked at random, and their ability to degrade MP or PNP was checked visually for the appearance and disappearance of yellow colour (indicative of PNP) in the culture medium. It was found that all the tested colonies could hydrolyse MP into PNP as shown by the appearance of yellow colour in the medium. However, 18 of them had lost the ability to degrade PNP (yellow colour did not disappear), while the others retained this activity (yellow colour disappeared). A degradation study further confirmed that plasmid-cured mutant (At) lost the PNP-degrading ability (Table 2). These results support loss of the plasmid as the basis for loss of PNP-degrading activity.

Discussion

The genus *Ochrobactrum* has been described by Holmes *et al.* (1988). *Ochrobactrum intermedium* (Velasco *et al.* 1998), *O. anthropi*, *Ochrobactrum gallinitefaesis*, *Ochrobactrum grinonense* and *Ochrobactrum tritici* (Lehuhn *et al.*

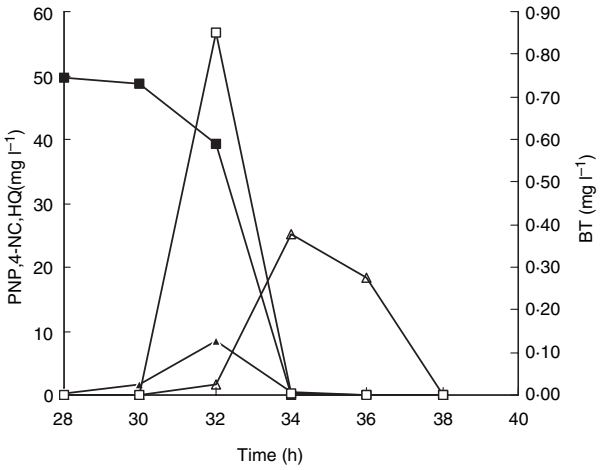

Figure 5 Quantification of *p*-nitrophenol depletion and formation of intermediates [4-nitrocatechol (4-NC), 1,2,4-benzenetriol (BT), hydroquinone (HQ)] by *Ochrobactrum* sp. B2 by HPLC analysis, when the ring cleavage was blocked using 2,2′-dipyridyl. (▲) 4-NC, (△) HQ, (■) *p*-nitrophenol, and (□) BT.

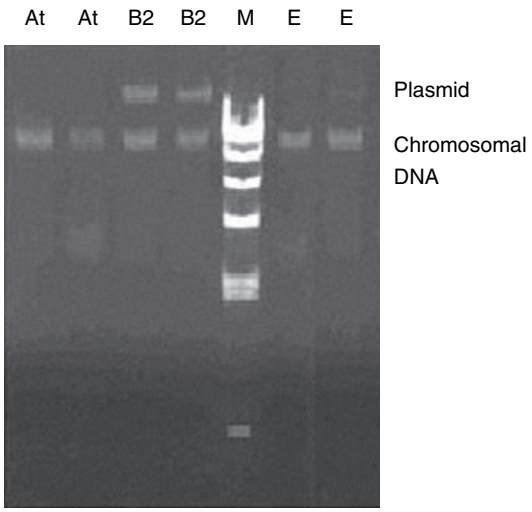

Figure 6 Agarose gel (0·8%) electrophoresis of plasmid DNA extracts from *Ochrobactrum* sp. B2 and its cured *p*-nitrophenol degradation-negative derivatives (At), and *Escherichia coli* Top 10 strain. Lane M is DNA marker of λDNA digested with *Hin*dIII (size of bands from top to bottom is 23 130, 9416, 6557, 4361, 2322, 2027, 564 bp respectively).

2000) have been reported as species belonging to this genus. *Ochrobactrum anthropi* strains, which degrade 4-chloro-2-methylphenol (Lechner *et al.* 1995), grew on plates containing atrazine as the only source of carbon (Laura *et al.* 1996), showed paraquat resistance (Won *et al.* 2000), and grew in the presence of lindane (Pesce and Wunderlin 2004). A halobenzoate-degrading bacterium related to *O. anthropi* was recently isolated and described (Bongkeuu *et al.* 2000). In addition, a strain with phenol-degrading activity which is closely related to *O. tritici* was isolated and identified by El-Sayed *et al.* (2003) from activated sludge for coke processing industrial waste treatment in Egypt. A very recent study investigating the diversity of MP-degrading bacteria in a contaminated soil in China revealed that four strains related closely to *O. tritici* had MP-degrading activity (Zhang *et al.* 2005). Our study provided a more conclusive case to show that strains of genus *Ochrobactrum* possess MP-degrading activity. These reports indicated that the genus *Ochrobactrum* may be involved in the metabolism of a range of xenobiotics.

Table 2 *p*-Nitrophenol (PNP) degradation by *Ochrobactrum* sp. (B2) and its plasmid-curing derivative (At)

	Residual PNP	
	After 0 h	After 36 h
Un-inoculated	22·56 ± 0·52	22·44 ± 1·36
B2	22·25 ± 2·26	Not detected
At	21·93 ± 1·03	21·92 ± 0·28

Data are the concentrations of PNP (mg l^{-1}) detected in the medium by HPLC. Results are mean values of two replicates ± standard error.

The degradation of MP by soil micro-organisms has been studied in various enrichment cultures. Generally, the initial step in organophosphate metabolism is the hydrolytic cleavage of the organophosphate bond (Rani and Lalithakumari 1994). Spectrophotometric analysis of the culture filtrate of B2 revealed the formation of PNP. Further evidence of its identity came from analyses by HPLC and GC-MS. The formation of PNP as a metabolite of MP degradation has been reported in *Pseudomonas* sp. (Chaudhry *et al.* 1988; Rani and Lalithakumari 1994; Ramanathan and Lalithakumari 1999; Liu *et al.* 2005), *Bacillus* sp. (Ou and Sharma 1989), *Plesiomonas* sp. (Cui *et al.* 2001) and other strains.

It is documented that PNP can be aerobically degraded by two major initial degradation pathways (Takeo *et al.* 2003; Kitagawa *et al.* 2004). One is called the HQ pathway, i.e. PNP is converted into HQ, and HQ is subsequently degraded via γ-hydroxymuconic semialdehyde and maleylacetate to β-ketoadipate (Spain and Gibson 1991; Rani and Lalithakumari 1994). This pathway was preferentially found in Gram-negative bacteria such as *Burkholderia* spp. and *Moraxella* spp. (Spain and Gibson 1991; Prakash *et al.* 1996). The other known pathway is called the 4-NC pathway, by which PNP is converted via 4-NC into BT, which was preferentially found in Gram-positive bacteria such as *Bacillus* spp. and *Arhtrobacter* spp. (Jain *et al.* 1994; Kadiyala and Spain 1998). Our

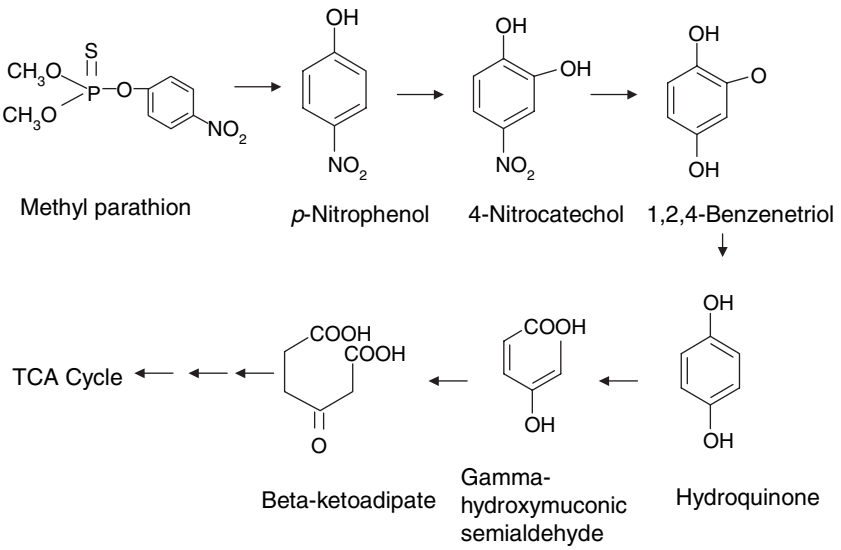

Figure 7 Proposed pathway for the degradation of *p*-nitrophenol by *Ochrobactrum* sp. B2.

studies showed that B2 degraded PNP with the release of nitrite ion and the formation of intermediates such as 4-NC, BT and HQ (Figs 3–5). From Fig. 5, we could find that HQ formed after 4-NC and BT. The results of intermediate identification and kinetics of appearance of these intermediates make us propose that MP could be degraded by B2 via MP → PNP → 4-NC → BT → HQ → → → TCA cycle (Fig. 7).

Our results showed that B2 can degrade MP, resulting from a rapid hydrolysis of this pesticide (Fig. 1), and its metabolite, PNP, was used as a carbon and energy source (Figs 1 and 2). The degradation of MP and its metabolite such as PNP by B2, and the fact that samples drawn after 72 h of growth did not show the presence of PNP and any other intermediates indicated the potential of this bacterium to completely mineralize MP. Until now, only a few reports of mineralizing MP by a single bacterial strain are available (Ramanathan and Lalithakumari 1999; Chen *et al.* 2002; Liu *et al.* 2005). The bioremediation potential of these isolates is worthy of attention for they could mineralize MP and a group of structurally related compounds.

The MP-hydrolysing gene may be chromosome- (Cui *et al.* 2001; Horne *et al.* 2002; Zhang *et al.* 2005) or plasmid-located (Liu *et al.* 2005). In most cases, PNP degradation activity was found to be plasmid-encoded (Prakash *et al.* 1996; Chauhan *et al* 2000). Recently, Liu *et al.* (2005) reported that genes coding for complete MP degradation is plasmid-borne in *Pseudomonas* sp. WBC-3 strain. In our study, the cured strain of B2 retained the MP-hydrolysing activity but simultaneously lost the PNP-degrading activity, strongly suggesting that the gene for MP hydrolysis in B2 was chromosomally located, while at least the initial PNP-degrading gene was plasmid-borne. We failed in our attempts to transform this plasmid chemically into *E. coli* in order to confirm its role in PNP degradation, and this becomes one subject of further investigation. Because we did not have large DNA markers available, the size of this plasmid needs to be determined. We have cloned a gene encoding MP hydrolysis activity by screening the genomic DNA library, and cloning and characterization of genes responsible for MP and PNP degradation is in progress in our laboratory.

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