

Biodegradation of methyl parathion and *p*-nitrophenol: evidence for the presence of a *p*-nitrophenol 2-hydroxylase in a Gram-negative *Serratia* sp. strain DS001

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Abstract A soil bacterium capable of utilizing methyl parathion as sole carbon and energy source was isolated by selective enrichment on minimal medium containing methyl parathion. The strain was identified as belonging to the genus *Serratia* based on a phylogram constructed using the complete sequence of the 16S rRNA. *Serratia* sp. strain DS001 utilized methyl parathion, *p*-nitrophenol, 4-nitrocatechol, and 1,2,4-benzenetriol as sole carbon and energy sources but could not grow using hydroquinone as a source of carbon. *p*-Nitrophenol and dimethylthiophosphoric acid were found to be the major degradation products of methyl parathion. Growth on *p*-nitrophenol led to release of stoichiometric amounts of nitrite and to the formation of 4-nitrocatechol and benzenetriol. When these catabolic intermediates of *p*-nitrophenol were added to resting cells of *Serratia* sp. strain DS001 oxygen consumption was detected whereas no oxygen consumption was apparent when hydroquinone was added to the resting cells suggesting that it is not part of the *p*-nitrophenol degradation

pathway. Key enzymes involved in degradation of methyl parathion and in conversion of *p*-nitrophenol to 4-nitrocatechol, namely parathion hydrolase and *p*-nitrophenol hydroxylase component “A” were detected in the proteomes of the methyl parathion and *p*-nitrophenol grown cultures, respectively. These studies report for the first time the existence of a *p*-nitrophenol hydroxylase component “A”, typically found in Gram-positive bacteria, in a Gram-negative strain of the genus *Serratia*.

Keywords Parathion hydrolase · *p*-Nitrophenol hydroxylase component A · *Serratia* sp · Biodegradation · Catabolomics

Introduction

Organophosphorus (OP) pesticides such as parathion, methyl parathion, and methamidophos are a group of highly toxic agricultural chemicals widely used in plant protection. As these pesticides cause enormous damage to nontarget organisms, their degradation has received considerable attention from soil microbiologists. Several OP pesticide-degrading bacteria have been isolated from agricultural soils in diverse geographical regions. Most of these bacteria possess a novel triesterase often referred to as parathion hydrolase or organophosphorus acid anhydrase and encoded by the highly conserved organophosphate-degradation (*opd*) gene, localized either on dissimilar indigenous plasmids or on the chromosome (Benning et al. 1994; Harper et al. 1988; Mulbry et al. 1986; Somara and Siddavattam 1995; Singh and Walker 2006; Zhang et al. 2005). Parathion hydrolase hydrolyses the characteristic triester bond found in a variety of OP pesticides including those present in nerve gas agents such as sarin and soman (Benning et al. 1994; Cho et al. 2004). Unlike the parent

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compounds, the hydrolytic products of OP compounds fail to inhibit acetyl cholinesterase (AChE) and, thus, have reduced toxicity towards higher animals with well-developed nervous systems. *p*-Nitrophenol (PNP) is one of the major hydrolytic products generated when the OP pesticides parathion and methyl parathion are subjected to microbial degradation (Chaudhry et al. 1988; Munnecke and Hsieh 1976; Rani and Lalithakumari 1994). Although PNP is less toxic to higher animals, it shows enhanced toxicity towards soil microflora (Errampalli et al. 1999). While several methyl parathion and PNP degrading bacteria have been isolated from OP-pesticide polluted soils, bacteria that simultaneously degrade both compounds are scarce (Liu et al. 2005; Ramanathan and Lalithakumari 1999; Rani and Lalithakumari 1994; Siddaramappa et al. 1973). Though there are preliminary reports of isolating parathion and *p*-nitrophenol degrading strains from flooded soils, detailed investigations pertaining to the degradation pathways are not available (Siddaramappa et al. 1973). In a fenitrothion degrading *Burkholderia* sp. the 3-methyl nitrophenol formed through initial hydrolysis is finally converted to hydroquinone before ring cleavage (Hayatsu et al. 2000). This process is similar to that in other Gram-negative bacteria, which typically convert PNP to maleylacetate via hydroquinone (Spain and Gibson 1991). In the present study, we report the isolation of a *Serratia* sp. strain (which we designated DS001) that can use both methyl parathion and PNP as sole source of carbon. We have shown that *Serratia* sp. strain DS001 degrades PNP to 4-nitrocatechol and 1,2,4-benzenetriol, the typical degradation products found when PNP is degraded by Gram-positive bacteria (Jain et al. 1994; Kadiyala and Spain 1998). Consistent with this activity, we have also demonstrated the existence of component “A” of a two-component *p*-nitrophenol hydroxylase in *Serratia* sp. strain DS001 which has otherwise only been found in Gram-positive bacteria.

Materials and methods

Isolation and taxonomic characterization of bacteria that degrade methyl parathion

Serratia sp. strain DS001 was isolated by an enrichment culture technique using agricultural soil from the Anantapur district of Andhra Pradesh in India that had a history of methyl parathion use in pest control activities. A 10-g soil sample was suspended in 30 ml of minimal medium (Chaudhry et al. 1988) containing 0.6 mM methyl parathion as sole source of carbon and incubated in a 250-ml flask at 30°C on an orbital shaker for a period of 7 days. After the incubation period, the soil particles were

allowed to settle and 5 ml of the particulate-free suspension was then used to inoculate a fresh 30-ml minimal medium containing methyl parathion (0.6 mM). Four such transfers were made and every time the enriched population was plated on minimal medium plates containing methyl parathion as sole carbon source. After the fourth transfer, a pure isolate capable of growth on methyl parathion was obtained.

The 16S rRNA genes of the isolate were amplified following procedures described elsewhere (Chen et al. 1997) and sequenced using BigDye polymerase (Applied Biosystems) followed by analysis on an ABI sequencer. This complete 16S rRNA gene sequence of the methyl parathion degrading bacterium (Genbank Acc. No. AM050059) was used to obtain related sequences from the NCBI database and among the sequences recovered only those that were approved by ATCC and DSMZ were selected for further study. These sequences were aligned using CLUSTALW to obtain percentage score values and nine full-length 16S rRNA gene sequences that showed 95 to 98% sequence similarity were selected to construct a phylogram using the CLUSTALX-BOOTSTRAP analysis program (Thompson et al. 1997).

Methyl parathion as a source of carbon or phosphorus

The growth behavior of *Serratia* sp. strain DS001 was monitored using methyl parathion as sole source of either carbon or phosphorus or both. Minimal medium [NH_4NO_3 , 1 g; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.2 g; $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$, 0.04 g; and $\text{Fe}_2(\text{SO}_4)_3$, 0.001 g per liter] (Chaudhry et al. 1988) was used with 0.6 mM methyl parathion as source of carbon and phosphorus. When required, 0.6 mM methyl parathion was supplemented as phosphorus source while using 2% filter-sterilized glucose as carbon source. Alternatively, a minimal medium [NH_4NO_3 , 1 g; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.2 g; $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$, 0.04 g; and $\text{Fe}_2(\text{SO}_4)_3$, 0.001 g per liter] used to grow *Serratia* sp. DS001 contained methyl parathion (0.6 mM) as carbon source, while K_2HPO_4 (4.8 g/l) and KH_2PO_4 (1.2 g/l) served as phosphate source. Growth of the cultures was monitored by measuring the optical density (OD) (600 nm) every 6 h.

p-Nitrophenol as a source of carbon

M9 medium (Maniatis et al. 1982) was used to monitor growth when PNP (0.3 mM) was used as a source of carbon. As NH_4Cl is used as nitrogen source in this medium, it was found to be highly suitable for monitoring release of nitrite during the oxidative degradation of PNP. Furthermore, to maintain uniformity, M9 medium was used in preference to minimal medium when using filter sterilized 4-nitrocatechol

(0.3 mM), hydroquinone (0.3 mM), or 1,2,4-benzenetriol (0.3 mM) as sole source of carbon. Nitrite released from the *p*-nitrophenol was determined following the method described by Barnes and Folkard (1951).

Oxygen uptake studies

Oxygen uptake studies by whole cells of *Serratia* sp. strain DS001 were conducted as described elsewhere using a Gilson Model 5/6 H oxygraph (Chauhan et al. 2000). Cultures were grown to mid log phase and cells from 5 ml culture were harvested and extensively washed with phosphate buffer pH 7.2. After washing, the cell pellet was dissolved in a minimal amount of phosphate buffer and appropriate amounts of cell suspension were added to a 1-ml reaction cell of the oxygraph to give a final OD of 0.6 at 600 nm. The oxygraph was stabilized to a baseline oxygen consumption and 50 μ mol of either PNP or its degradation products, such as 4-nitrocatechol or hydroquinone, were added to the reaction cell. Specific activities were expressed as nmol of O₂ consumed per minute per milligram of protein. Benzenetriol stock solution was prepared in water adjusted to pH 2.0 using dilute HCl and appropriate amounts of stock solution was added to the cells suspended in 50 mM MOPS buffer. MOPS buffer was used for suspending the cells to prevent autooxidation of benzenetriol.

Characterization of metabolites by GC-MS analysis

The degradation products of methyl parathion and PNP were extracted from spent medium by following the standard procedures (Prakash et al. 1996) and analyzed using GCMS-QP 5050A (Shimadzu), essentially by following the protocols developed by Rani and Lalithakumari (1994) with slight modifications. In the present study, we used a column (25 m \times 0.2 mm ID \times 0.33 μ) packed with 100% SPB1, Supelco (Sigma). The column, injector port, and detector temperatures were maintained at 210, 230, and 250°C, respectively. Helium was used as the carrier gas and maintained at the flow rate of 1 ml/min. The degradation products of methyl parathion and PNP were identified by comparing retention times and MS data of the unknown with those of reference compounds.

Two-dimensional gel electrophoresis

Serratia sp. strain DS001 was grown to log phase in M9 media supplemented either with methyl parathion or PNP as sole source of carbon. Cells grown in M9 medium with glucose as carbon source served as a control. Log phase cells were collected and the cell pellet was washed three times with the low salt buffer (3 mM KCl, 1.5 mM

KH₂PO₄, 68 mM NaCl, and 9 mM NaH₂PO₄). Finally, the pellet was resuspended in 0.2 ml Tris containing 5 mM MgCl₂, 5 U of DNase and RNase and 20 μ l of a solubilization solution containing 20% CHAPS, 10% DTT, and 20% ampholytes. Cells were then sonicated three times for 1 min at power setting of 5 and 50% pulse at 4°C and the sonicate was finally centrifuged at 25,000 \times g at 4°C for 30 min. Proteins were quantified by the Bradford method (Bradford 1976) and 100 μ g of protein was treated with the Amersham 2D clean-up kit to remove the salts (Duffes et al. 2000) and loaded onto isoelectrofocusing (IEF) 17 cm, pH 3–10 IPG strips (Biorad) and focussed using an IEF (Biorad) cell. The strips were then equilibrated with Biorad equilibration buffer I for 1 h. The equilibrated strips were placed on 10% acrylamide gels and electrophoresis was carried out at 200 V until completion. Gels were fixed in 40% methanol, 10% acetic acid overnight, silver stained and scanned with a densitometer scanner (Biorad).

In-gel digestion/mass spectrometry (MALDI-TOF) analysis

The silver stained protein band was excised manually, chopped into small pieces and transferred into Eppendorf tubes. A piece of protein-free acrylamide gel was taken in parallel as a negative control. The gel pieces were then destained, water washed, and reduced for 1 h at 57°C using reduction buffer [100 mM NH₄HCO₃, 10 mM dithiothreitol (DTT)] to disrupt disulfide bonds. The bands were alkylated using alkylation buffer (100 mM NH₄HCO₃ and 55 mM iodoacetamide) and were placed in the dark for 30 min at room temperature to prevent disulfide bonds from reforming (alkylation). The gel pieces were dehydrated with 50 μ l of 100% acetonitrile, and then dried under vacuum using a Speedvac concentrator for 30 min.

Digestion solution (100 ng/ μ l trypsin) in 50 mM of NH₄HCO₃ was added to the dried gel pieces which were submerged in the digestion solution of 40 μ l and incubated overnight at 37°C. Peptides were extracted from the digested mixture by adding 100 μ l of extraction buffer (50% acetonitrile containing 5% trifluoroacetic acid). This process was repeated twice and the supernatant was collected after spinning at 10,000 g. The resulting peptide mixture was further concentrated in a Speedvac concentrator for 1 h. The extracted peptide mixture was desalted using Millipore Ziptip C-18 columns. The desalted tryptic peptide mixture was mixed with a cyano-4-hydroxycinnamic acid (CHCA) matrix solution (CHCA matrix dissolved in 50% acetonitrile having 0.1% trifluoroacetic acid of 1 μ l) and vortexed gently. A volume of 2 μ l of the mixture containing CHCA matrix and the tryptic digest were loaded on a stainless steel plate and air-dried. Proper

care was taken to prevent any keratin contamination. The sample was analyzed by Micromass MALDI-TOF [Model MALDI-R (reflectron mode) Serial No. E RA076] in reflectron mode. A pulsed nitrogen laser of 337 nm was fired (voltage conditions: pulse voltage 3,120; source voltage 15,000; and reflectron voltage 500) to accumulate 100 shots per spectra and the peptide mass finger print profile of the sample was generated. The spectra width was narrowed to a range from 500 to 3,500 Da m/z . The spectra were processed (baseline correction, noise removal, deisotoping) by using Mass Lynx 3.5 version software. Proteins were identified using the public domain Mascot search engine by incorporating the standard parameters (<http://www.matrixscience.com>). The database used was Swiss-prot and trypsin was used as proteolytic enzyme by limiting the miscleavages to one. Carbamidomethyl (C) and oxidation of methionine were taken as fixed and variable modifications, respectively. The peptide mass values were MH⁺ and monoisotopic and mass tolerance was limited to 60 ppm.

Parathion hydrolase assay

Cells of *Serratia* sp. strain DS001 that were in mid log phase were harvested and the cell pellet was extensively washed with citrate saline buffer to remove traces of methyl parathion. The cell pellet was finally resuspended in 5 ml of 10 mM Tris HCl buffer, pH 7.6 containing lysozyme (500 $\mu\text{g/ml}$). The cells were disrupted using a sonicator (10 cycles at 8,000 amplitude, 1.5 min per cycle with a 1-min interval). The cell lysate was briefly centrifuged at 5,000 rpm to remove unbroken cells and the supernatant obtained was used to obtain cytosolic and membrane fractions by subjecting it to ultra centrifugation at 200,000 $\times g$ for 45 min. The crude extract, membrane and cytoplasmic fractions were used to assess parathion hydrolase activity by following standard procedures (Chaudhry et al. 1988). Parathion hydrolase activity was expressed as nanomoles of PNP formed per minute per milligram of protein.

Induction and assay of 4-nitrophenol 2-hydroxylase

Cells of *Serratia* sp. strain DS001 were grown in minimal medium containing reduced concentrations of glucose (10 g/l) for 16 h before harvesting and resuspending them in induction medium. The induction medium was prepared by dissolving 3 g Na₂HPO₄, 0.3 g KH₂PO₄, 0.3 g MgSO₄·7H₂O, 1 g NH₄Cl, 0.001 g FeSO₄ in 1 l of distilled water and adjusting the pH to 7.3. The cells were grown in induction medium, to which 100 μmol PNP was added per 50 ml of medium, for 8 h with additional amounts (100 μmol) of PNP added every 2 h. After 8 h, the cells were harvested and fractionated into

cytoplasmic and membrane fractions as described above. The 4-nitrophenol 2-hydroxylase assay was performed by measuring the increase in absorbance at 510 nm due to formation of 4-nitrocatechol (Mitra and Vaidyanathan 1984).

Results

Isolation and taxonomic characterization of strain DS001

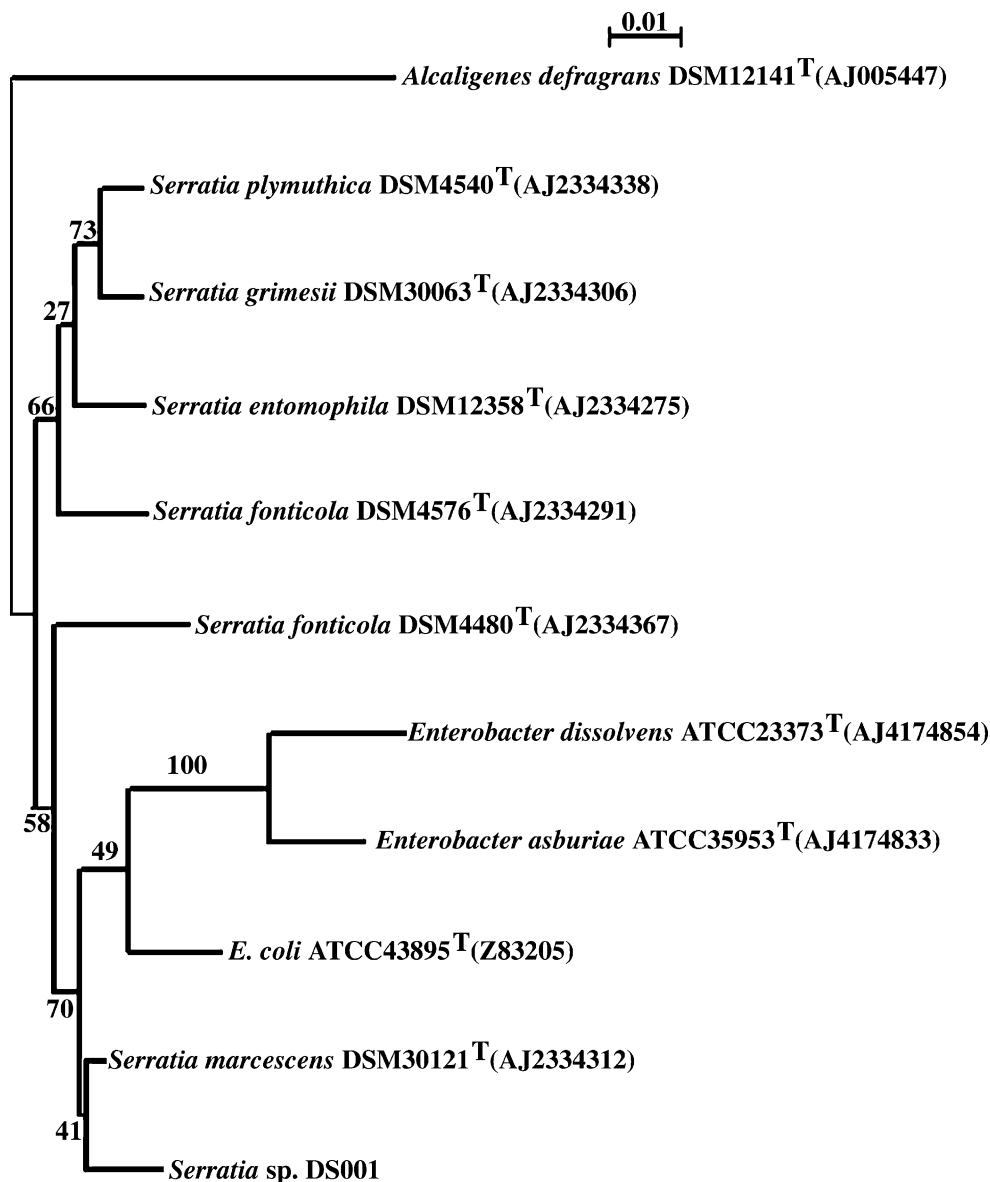
The rod-shaped bacterial strain DS001 isolated from agricultural soils was Gram-negative. Strain DS001 besides showing catalase and cytochrome oxidase activity also possessed the ability to reduce nitrites. When the complete 16S rRNA sequence was used to establish a phylogram, this revealed that strain DS001 was most closely related (98% homology) to *Serratia marcescens* DSM30121. The phylogenetic tree constructed for comparing the outgroup clearly indicated that the isolate belongs to the γ proteobacteria and the genus *Serratia* and, hence, it was designated *Serratia* sp. strain DS001 (Fig. 1). The culture has been deposited in the Microbial Culture Collection Centre, Institute of Microbial Technology, Chandigarh, India and assigned the accession no. MTCC 4830.

Growth behavior of *Serratia* sp. strain DS001

Serratia sp. strain DS001 effectively utilized methyl parathion as sole source of carbon and phosphorus. Although it was capable of growth in minimal medium containing 2.25 mM methyl parathion, maximum growth was observed at a concentration of 0.75 mM. There was an initial lag in growth when methyl parathion was used as a phosphate source (data not shown) but no such lag phase was apparent when it was supplemented as a source of carbon (Fig. 2). In both cases, maximum growth was recorded 24 h after inoculation and the culture reached stationary phase after 30 h. With methyl parathion as the carbon source, the doubling time was 10.5 h. But when used as both the source of carbon and phosphorus, the doubling time was 22 h. During growth of the culture, the medium turned yellow due to hydrolysis of methyl parathion and formation of PNP and the intensity of the yellow color increased proportionately with the growth of the culture. However, after 18 h of incubation, there was a decrease in the concentration of PNP suggesting that *Serratia* sp. strain DS001 was also able to use PNP as a source of carbon.

The prime objective of this study was to establish whether the pathway involved complete mineralization of methyl parathion and, therefore, we attempted to grow the cells on possible catabolic intermediates of methyl parathion. As PNP is a major recalcitrant catabolic intermediate of

Fig. 1 Phylogram showing the taxonomic placement of *Serratia* sp. DS001 among reference species of the γ -proteobacteria class. Bootstrap values for 100 trees are shown at branch points

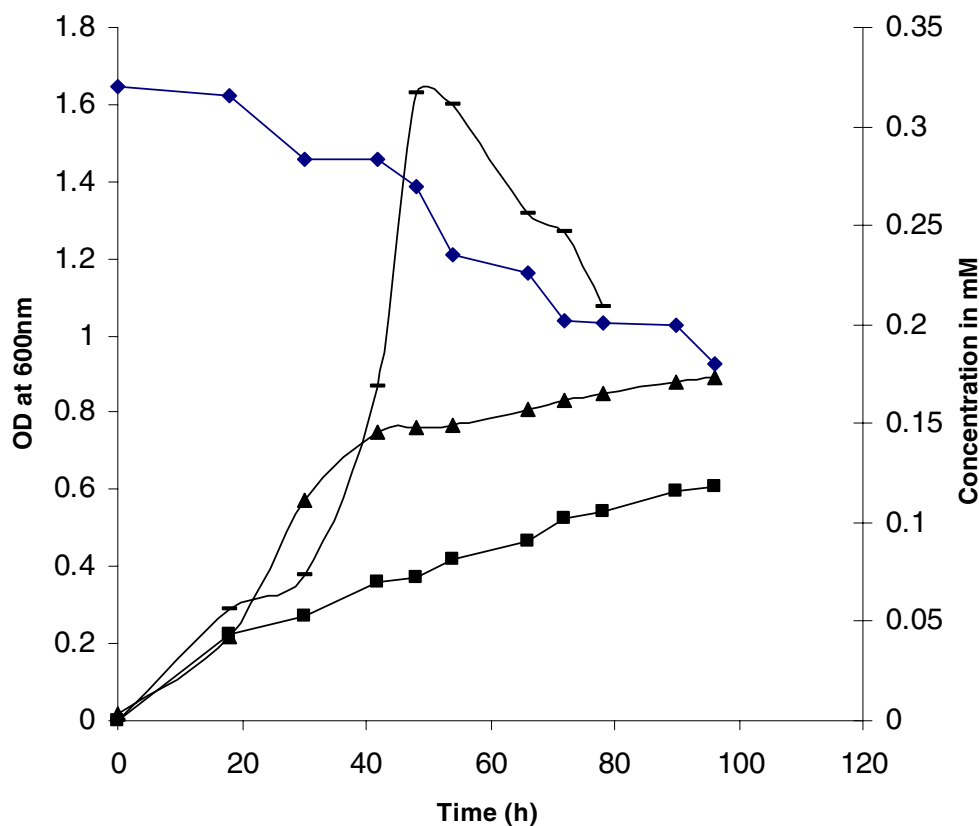


methyl parathion degradation, we attempted to grow the cells in minimal medium supplemented with PNP. Though growth was recorded at concentrations as high as 4 mM, optimum growth was found at a concentration of 0.3 mM PNP when the doubling time was 13.8 h. *Serratia* sp. strain DS001 effectively degraded PNP such that around 30% of the available PNP was utilized in 70 h (Fig. 2). When PNP was used as sole source of carbon, stoichiometric amounts of nitrite were also released into the medium concomitant with the degradation of PNP (Fig. 2) suggesting the involvement of an initial oxidative step in PNP degradation.

The oxidative degradation of PNP is well established both in Gram-positive and Gram-negative bacteria (Jain et

al. 1994; Kadiyala and Spain 1998; Spain and Gibson 1991; Takeo et al. 2003). Therefore, the growth behavior of *Serratia* sp. strain DS001 was examined using known catabolic intermediates of PNP as sole source of carbon. 4-Nitrocatechol and 1,2,4-benzenetriol are reported to be the major degradation products when the PNP is degraded by Gram-positive bacteria (Jain et al. 1994; Kadiyala and Spain 1998; Kitagawa et al. 2004) and benzoquinone was found to be the key catabolic intermediate when PNP was degraded by Gram-negative bacteria (Prakash et al. 1996; Spain and Gibson 1991). *Serratia* sp. strain DS001 was able to use 4-nitrocatechol and 1,2,4-benzenetriol but not hydroquinone as sole carbon source suggesting that PNP, 4-nitrocatechol and 1,2,4-benzenetriol are likely intermedi-

Fig. 2 Growth of *Serratia* sp. strain DS001 when either 0.6 mM methyl parathion (straight line) or 0.3 mM *p*-nitrophenol (filled triangle) is used as source of carbon. PNP utilization and nitrite concentration is shown either with filled squares or diamonds



ates in the degradation of methyl parathion. Although dimethyl thiophosphoric acid is also expected to form due to the hydrolysis of methyl parathion, its utilization as a carbon source could not be confirmed by growth studies due to nonavailability of a commercial source.

Oxygen uptake studies

After examining the growth behavior of *Serratia* sp. strain DS001 on methyl parathion and its catabolic intermediates we further tried to establish the relationship between the rate of degradation of PNP and its catabolic intermediates and consumption of oxygen. Strain DS001 was grown in minimal medium supplemented with either methyl parathion, PNP, or glucose. When the cell suspension prepared from the methyl parathion grown cultures was mixed with either PNP or its catabolic intermediates 4-nitrocatechol or 1,2,4-benzenetriol, there was a significant increase in the oxygen uptake. When the oxygen consumption was quantified, it was found to be $44.9 \text{ nmol min}^{-1} \text{ mg}^{-1}$ (PNP), $48.5 \text{ nmol min}^{-1} \text{ mg}^{-1}$ (4-nitrocatechol), and $56.6 \text{ nmol min}^{-1} \text{ mg}^{-1}$ (1,2,4-benzenetriol) of protein for methyl parathion grown cultures. A similar trend was observed in the oxygen consumption pattern of cultures that were grown in PNP. In marked comparison, glucose grown cells showed no oxygen consumption when either

PNP or 1,2,4-benzenetriol were added and only low levels of oxygen consumption when 4-nitrocatechol was added, indicating the inducible nature of the PNP degrading enzymes. No significant oxygen consumption was apparent when hydroquinone was mixed with cell suspensions of methyl parathion-grown or PNP-grown cells; thus, strengthening the data obtained in growth studies where hydroquinone failed to serve as source of carbon.

GCMS analysis of metabolic intermediates of methyl parathion and *p*-nitrophenol

GCMS analysis was used to identify further the catabolic intermediates of methyl parathion and PNP degradation and several characteristic peaks that are identical to well-characterized authentic compounds were observed (Supplementary Figs. 1, 2, 3, and 4). Compounds extracted from the spent medium containing methyl parathion as source of carbon showed the presence of PNP with a retention time of 19.95 min. The molecular ion $[M^+]$ at m/z 139 corresponding to the mass and major fragmentation ions was found to be identical to the mass spectral properties of the authentic PNP. Similarly, a second characteristic peak with a retention time of 8.9 min and a molecular ion $[M^+]$ at m/z 141 showed identity with the mass of dimethylthiophosphoric acid, suggesting the existence of a hydrolytic step

involving phosphotriesterase in the degradation of methyl parathion. Furthermore, the catabolic intermediates of PNP, 4-nitrocatechol, and benzenetriol were also identified based on retention time and mass spectral data: 4-nitrocatechol, retention time 21.1 min, and molecular ion $[M^+]$ at m/z 155; benzenetriol, retention time 19.0 min, and molecular ion $[M^+]$ at m/z 126 (Chauhan et al. 2000).

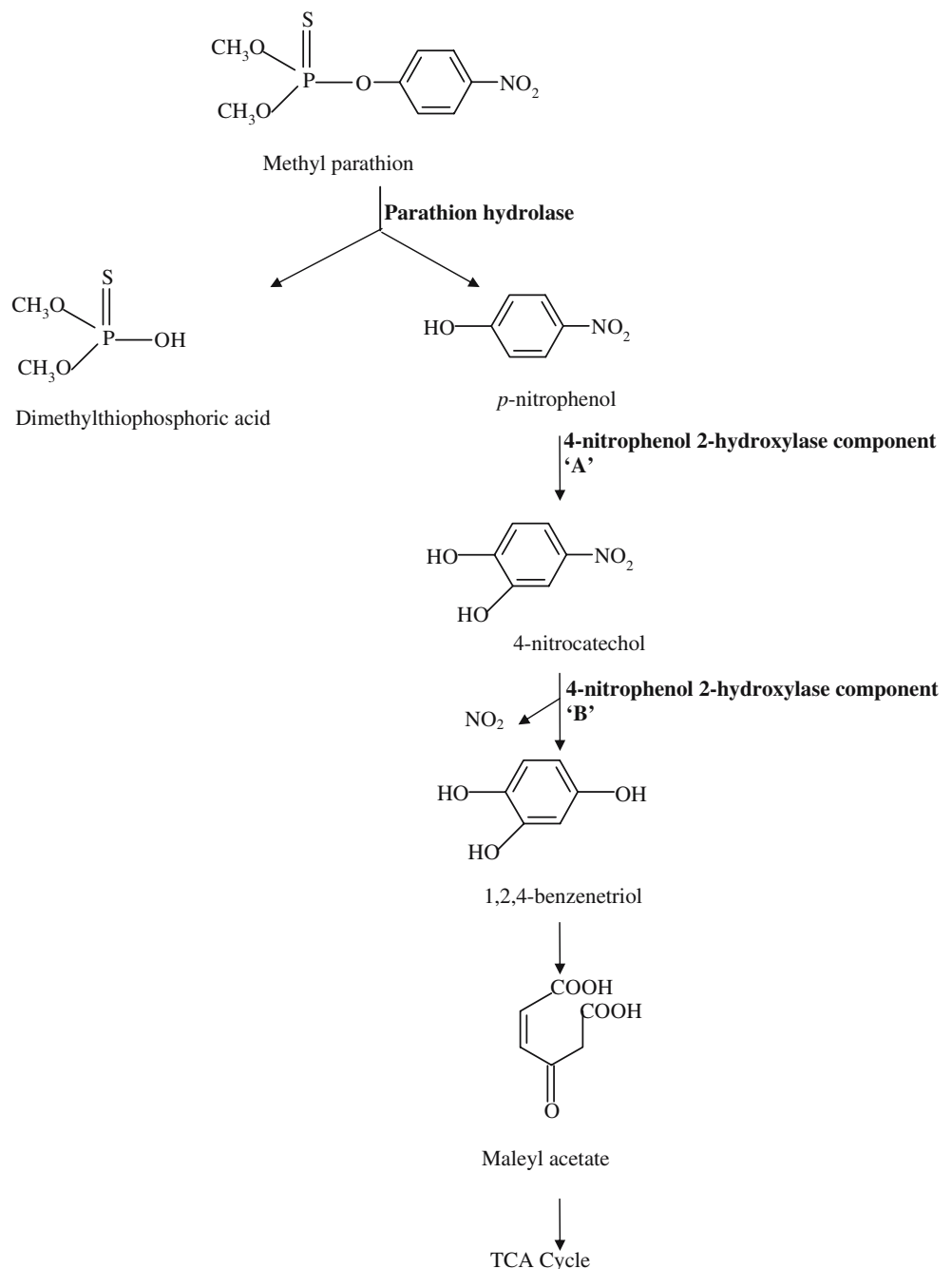
These data support the conclusion that methyl parathion can be degraded completely by *Serratia* sp. strain DS001. It appears that the process of mineralization of methyl parathion is initiated by hydrolysis leading to the generation of PNP

and dimethylthiophosphoric acid and that PNP degradation then proceeds through formation of 4-nitrocatechol and 1,2,4-benzenetriol. These observations suggest a likely pathway for the degradation of methyl parathion (Fig. 3).

Enzyme assays

To support this proposed degradation pathway, we assayed key enzymes involved in the initial hydrolysis of methyl parathion and the conversion of PNP to 4-nitrocatechol. Previous investigations involving the degradation of methyl

Fig. 3 Proposed degradation pathway of methyl parathion in *Serratia* sp. strain DS001



parathion revealed the existence of a well-characterized triesterase (designated parathion hydrolase or phosphotriesterase) in OP-compound degrading bacteria. We therefore measured parathion hydrolase activity in *Serratia* sp. strain DS001 cells grown with either methyl parathion or glucose as carbon source. There was no activity in glucose-grown cells (data not shown). In cells grown in methylparathion, a major component of parathion hydrolase activity, about 65% activity ($1,700 \text{ nmol min}^{-1} \text{ mg}^{-1} \text{ protein}$), was found in the cellular membrane fraction with the rest, 35% ($892 \text{ nmol min}^{-1} \text{ mg}^{-1} \text{ protein}$), in the cytoplasmic fraction. We also measured the activity of 4-nitrophenol 2-hydroxylase, the key enzyme involved in conversion of *p*-nitrophenol to 4-nitrocatechol (Mitra and Vaidyanathan 1984). Nearly 73% of the 4-nitrophenol 2-hydroxylase activity ($24 \text{ nmol min}^{-1} \text{ mg}^{-1} \text{ protein}$) was found in the membrane fraction with the remaining 27% activity ($8.9 \text{ nmol min}^{-1} \text{ mg}^{-1} \text{ protein}$) in the cytoplasm, indicating that 4-nitrophenol 2-hydroxylase was also associated with the membrane.

Catabolomics of methyl parathion degradation

Additional data to aid elucidation of the degradation pathway was derived by analysis of the proteome of cells grown on methyl parathion or PNP with the intention of identifying the key enzymes involved in pathway. The proteomes expressed in *Serratia* sp. strain DS001 when grown in glucose, methyl parathion, or PNP were extracted and analyzed by two-dimensional electrophoresis (2DE). Approximately 300 protein spots were detected on each 2DE gel using a pH 3–10 gradient and 10% polyacrylamide sodium dodecyl sulfate gels (Supplementary Fig. 5). Comparison of the 2DE gels prepared from the proteome of methyl parathion-grown or PNP-grown cells with the proteome of glucose-grown cells revealed several proteins that were differentially expressed according to the growth medium. However, in addition to those proteins, eight protein spots on 2DE gels from methyl parathion-grown cells and 10 protein spots from PNP-grown cells were novel and their counterparts were not found in glucose-grown cultures. In the present study, we concentrated only on these protein spots and established their identity by

MALDI-TOF. The proteins identified are listed in Tables 1 and 2. Most significantly, we identified two important enzymes involved in degradation of methyl parathion: one of them is the precursor form of parathion hydrolase (Serdar et al. 1989) in which the signal peptide encoded by the first 29 amino acids has not yet been removed, and the other is component “A” of 4-nitrophenol 2-hydroxylase, the enzyme involved in conversion of PNP to 4-nitrocatechol.

In summary, all of the data described above are consistent with a degradation pathway for methylparathion in *Serratia* sp. strain DS001 as depicted in Fig. 3.

Discussion

Serratia sp. strain DS001 was found to degrade both methyl parathion and its major degradation product *p*-nitrophenol (PNP). The initial hydrolytic step was found to be due to the presence of a well-characterized phosphotriesterase known as parathion hydrolase (Mulbry et al. 1986). The identification of the precursor form of parathion hydrolase among the proteome of parathion grown cultures and detection of dimethylthiophosphoric acid and PNP from the spent medium of a methyl parathion-grown culture confirm the existence of a parathion hydrolase homologue in *Serratia* sp. strain DS001. Bacterial parathion hydrolases are encoded by the highly conserved organophosphate degradation (*opd*) gene found on large, dissimilar, and indigenous plasmids (Harper et al. 1988; Mulbry et al. 1986; Somara and Siddavattam 1995). However the *opd* genes reported to date are constitutively expressed (Mulbry and Karns 1989), whereas in DS001 the precursor form of parathion hydrolase and parathion hydrolase activity were only detected in methyl parathion-grown cultures and not in glucose-grown or PNP-grown cultures. Hence, it appears that parathion hydrolase is an inducible enzyme in *Serratia* sp. strain DS001. We only identified the precursor form (before signal peptide cleavage) and not the mature form of parathion hydrolase in the proteome of methyl parathion grown DS001. However, parathion hydrolase has been reported to be membrane-associated in other organisms (Brown 1980;

Table 1 Proteins identified uniquely in the proteome extracted from methyl parathion grown *Serratia* sp. strain DS001

Spot number	Mw	pI	Protein	Swiss-Prot accession number
1	39	8.0	Parathion hydrolase	POA433
2	27	5.5	Glutathione S-transferase	P82999
3	37	7.2	Maleylacetate reductase	O84992
4	36	4.2	Ferrochelatase	P43413
5	36	8.0	Galactokinase	P39574
6	31	4.3	Pantoate β -alanine ligase	Q8KBY5
7	36	4.5	ATP phosphoribosyl transferase	Q8X8T4
8	35	5.0	γ -glutamyl phosphate reductase	Q8YQ41

Table 2 Proteins identified uniquely in the proteome extracted from PNP grown *Serratia* sp. strain DS001

Spot number	Mw	pI	Protein	Swiss-Prot accession number
1	57	6.25	<i>p</i> -Nitrophenol hydroxylase component A	Q8RQQ0
2	51.7	5.95	2-Hydroxymuconic semialdehyde dehydrogenase	P23105
3	90	6.42	DMSO reductase	P18775
4	45	6	Aromatic amino acid aminotransferase	P95468
5	47	3.8	Histidinol dehydrogenase	P06988
6	45	6	Adenosyl methionine synthetase	Q6FAQ6
7	28	5	GTP cyclohydrolase I	Q8A0U0
8	32	4	Bisphosphoglycerate dependent phosphoglycerate mutase	Q737X5
9	45	4.8	Glutamate <i>N</i> -acetyl transferase	P62059
10	25	4.5	<i>N</i> -acetyl glucosaminyl transferase	Q53513

McDaniel et al. 1988). Consistent with this, we detected the majority of this activity in the membrane fraction of *Serratia* sp. strain DS001 and the activity in the cytoplasm could be due to the relatively weak membrane association of the protein or to some activity of the precursor form. As the cell lysate was subjected to ultracentrifugation before 2DE analysis, the mature form of the enzyme might be expected to have been lost from the samples detected in the proteome.

The oxidative degradation of *p*-nitrophenol by DS001 was found to be due to initial hydroxylation by 4-nitrophenol 2-hydroxylase. Several lines of evidence support the formation of 4-nitrocatechol due to 4-nitrophenol 2-hydroxylase activity. GCMS data and oxygen uptake studies supported the formation of 4-nitrocatechol and 1, 2, 4-benzenetriol by oxidative degradation of *p*-nitrophenol, while none of our studies indicated formation of hydroquinone, a typical catabolic intermediate of PNP in Gram-negative bacteria. The catabolome of PNP grown cultures clearly showed the presence of *p*-nitrophenol hydroxylase, which is responsible for generation of 4-nitrocatechol from PNP. The protein showed homology to component “A” of the two-component PNP hydroxylase of *Rhodococcus* sp. PN1 (Takeo et al. 2003). The two-component monooxygenases, though rare, are not uncommon among prokaryotes. The existence of a two-component PNP monooxygenase has been reported in Gram-positive *Bacillus sphaericus* JS 905 (Kadiyala and Spain 1998) and *Rhodococcus opacus* SAO101 (Kitagawa et al. 2004). However, although there are several reports of the existence of 4-nitrophenol 4-hydroxylases that generate hydroquinone from PNP, the presence of a 4-nitrophenol 2-hydroxylase that converts PNP into 4-nitrocatechol is not very common in Gram-negative bacteria. We are aware of only two reports where *p*-nitrophenol is converted to nitrocatechol by a Gram-negative organism (Leung et al. 1997; Roldan et al. 1998). It is interesting to note that in *Spingomonas* sp. UG30 the pentachlorophenol (PCP) 4-monooxygenase,

which is generally involved in hydroxylation of pentachlorophenols at the *para*-position, was shown to convert nitrocatechol to benzenetriol (Leung et al. 1999). In vitro studies of PCP monooxygenase showed very little activity with *p*-nitrophenol, which led the authors to speculate on the presence of a separate monooxygenase responsible for the conversion of *p*-nitrophenol to nitrocatechol (Cassidy et al. 1999; Leung et al. 1999). However there is no experimental evidence to substantiate this idea. Similarly in *Rhodobactercapsulatus* nitrocatechol formed from PNP is directly converted to 4-nitroketo adipate. As seen in Gram-positive bacteria, benzenetriol is not part of the PNP degradation pathway (Roldan et al. 1998). Indeed to our knowledge, our data on *Serratia* sp. strain DS001 constitute the first report showing a PNP degradation pathway involving a *p*-nitrophenol 2-hydroxylase in a Gram-negative species. As these results are novel, we have tried to ensure the unambiguous taxonomic status of the *Serratia* sp. strain DS001 by comparing the complete sequence of the 16S rRNA with the similar sequences of well-characterized type strains. The phylogram based on this analysis showed 98% similarity to the Gram-negative γ proteobacterium *Serratia marsicens*.

In the proteome extracted from methyl parathion grown cultures, several other proteins in addition to parathion hydrolase were exclusively expressed (Table 2). Of particular interest was maleylacetate reductase which is a key player in channeling maleylacetate and its substituted derivatives in to the β -keto adipate pathway (Kadiyala and Spain 1998; Muller et al. 1996). There are number of reports in the literature that support formation of maleylacetate from benzenetriol due to the action of benzenetriol dioxygenases (Daubaras et al. 1996; Kadiyala and Spain 1998; Kitagawa et al. 2004). Hence, the presence of maleylacetate reductase further supports formation of benzenetriol (Fig. 3), which is the key product, formed from nitrocatechol due to the activity of two-component monooxygenase (Kadiyala and Spain 1998). Similarly, in

the proteome of PNP-grown cultures, in addition to PNP-4-nitrophenol 2-hydroxylase component A, nine other proteins, the majority of which are involved in either de novo synthesis or degradation of amino acids (Loper 1968; Weigent and Nester 1976), were found uniquely in this growth condition (Table 2). One among them is 2-hydroxy muconic semialdehyde dehydrogenase, generally found to act on *meta*-fission product of catechol (Murray et al. 1972). This enzyme does not seem to have a direct role in degradation of *p*-nitrophenol. As *meta*-fission product hydrolases and dehydrogenases are nonspecifically induced (Murray et al. 1972), the presence of this enzyme in *Serratia* sp. strain DS 001 that can also grow on benzoic acid and catechol (data not shown) is not surprising. The possible physiological significance of the other proteins exclusively found in specific growth conditions remains to be established.

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References

- Barnes H, Folkard AR (1951) The determination of nitrites. *Analyst* 76:599–603
- Benning MM, Kuo JM, Raushel FM, Holden HM (1994) Three-dimensional structure of phosphotriesterase: an enzyme capable of detoxifying organophosphate nerve agents. *Biochemistry* 33:15001–15007
- Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 72:248–254
- Brown KA (1980) Phosphotriesterases of *Flavobacterium* sp. *Soil Biol Biochem* 12:105–112
- Cassidy MB, Lee H, Trevors JT, Zablutowicz RB (1999) Chlorophenol and nitrophenol metabolism by *Sphingomonas* sp. UG 30. *J Ind Microbiol Biotechnol* 23:232–241
- Chaudhry GR, Ali AN, Wheeler WB (1988) Isolation of a methyl parathion-degrading *Pseudomonas* sp. that possesses DNA homologous to the *opd* gene from a *Flavobacterium* sp. *Appl Environ Microbiol* 54:288–293
- Chauhan A, Samanta SK, Jain RK (2000) Degradation of 4-nitro-catechol by *Burkholderia cepacia*: a plasmid-encoded novel pathway. *J Appl Microbiol* 88:764–772
- Chen WX, Tan ZY, Gao JL, Li Y, Wang ET (1997) *Rhizobium hainanense* sp. nov., isolated from tropical legumes. *Int J Syst Bacteriol* 47:870–873
- Cho CM, Mulchandani A, Chen W (2004) Altering the substrate specificity of organophosphorus hydrolase for enhanced hydrolysis of chlorpyrifos. *Appl Environ Microbiol* 70:4681–4685
- Daubaras DL, Saido K, Chakrabarty AM (1996) Purification of Hydroxyquinol 1,2-dioxygenase and Maleylacetate reductase: the lower pathway of 2,4,5-trichlorophenoxyacetic acid metabolism by *Burkholderia cepacia* AC1100. *Appl Environ Microbiol* 62:4276–4279
- Duffes F, Jenoe P, Boyaval P (2000) Use of two-dimensional electrophoresis to study differential protein expression in divercin V41-resistant and wild-type strains of *Listeria monocytogenes*. *Appl Environ Microbiol* 66:4318–4324
- Errampalli D, Tresse O, Lee H, Trevors JT (1999) Bacterial survival and mineralization of *p*-nitrophenol in soil by green fluorescent protein-marked *Moraxella* sp. G21 encapsulated cells. *FEMS Microbiol Ecol* 30:229–236
- Harper LL, Mcdaniel CS, Miller CE, Wild JR (1988) Dissimilar plasmids isolated from *Pseudomonas diminuta* MG and a *Flavobacterium* sp. (ATCC 27551) contain identical *opd* genes. *Appl Environ Microbiol* 54:2586–2589
- Hayatsu M, Hirano M, Tokuda S (2000) Involvement of two plasmids in Fenitrothion degradation by *Burkholderia* sp. strain NF100. *Appl Environ Microbiol* 66:1737–1740
- Jain RK, Dreisbach JH, Spain JC (1994) Biodegradation of *zp*-nitrophenol via 1,2,4-benzenetriol by an *Arthrobacter* sp. *Appl Environ Microbiol* 60:3030–3032
- Kadiyala V, Spain JC (1998) A two-component monooxygenase catalyzes both the hydroxylation of *p*-nitrophenol and the oxidative release of nitrite from 4-nitrocatechol in *Bacillus sphaericus* JS905. *Appl Environ Microbiol* 64:2479–2484
- Kitagawa W, Kimura N, Kamagata Y (2004) A novel *p*-nitrophenol degradation gene cluster from a gram-positive bacterium, *Rhodococcus opacus* SAO101. *J Bacteriol* 186:4894–4902
- Leung KT, Tresse O, Errampalli D, Lee H, Trevors JT (1997) Mineralization of *p*-nitrophenol by pentachlorophenol-degrading *Sphingomonas* spp. *FEMS Microbiol Lett* 155:107–114
- Leung KT, Campbell S, Gan Y, White DC, Lee H, Trevors JT (1999) The role of the *Sphingomonas* species UG 30 pentachlorophenol-4-monooxygenase in *p*-nitrophenol degradation. *FEMS Microbiol Lett* 173:247–253
- Liu H, Zhang J, Wang S, Zhang X, Zhou N (2005) Plasmid-borne catabolism of methylparathion and *p*-nitrophenol in *Pseudomonas* sp. strain WBC-3. *Biochem Biophys Res Commun* 334:1107–1114
- Loper JC (1968) Histidinol dehydrogenase from *Salmonella typhimurium*. Crystallization and composition studies. *J Biol Chem* 243:3264–3272
- Maniatis T, Fritsch EF, Sambrook J (1982) Molecular cloning. A laboratory manual. Cold Spring Harbor Laboratory Press, New York
- Mcdaniel CS, Harper LL, Wild JR (1988) Cloning and sequencing of a plasmid-borne gene (*opd*) encoding a phosphotriesterase. *J Bacteriol* 170:2306–2311
- Mitra D, Vaidyanathan CS (1984) A new 4-nitrophenol 2-hydroxylase from a *Nocardia* sp. *Biochem Int* 8:609–615
- Mulbry WW, Karns JS (1989) Purification of three parathion hydrolases from gram-negative bacterial strains. *Appl Environ Microbiol* 55:289–293
- Mulbry WW, Karns JS, Kearney PC, Nelson JO, Mcdaniel CS, Wild JR (1986) Identification of a plasmid-borne parathion hydrolase gene from *Flavobacterium* sp. by southern hybridization with *opd* from *Pseudomonas diminuta*. *Appl Environ Microbiol* 51:926–930
- Muller DM, Schlomann M, Reineke W (1996) Maleylacetate reductases in chloroaromatic-degrading bacteria using the modified ortho pathway: comparison of catalytic properties. *J Bacteriol* 178:298–300
- Munnecke DM, Hsieh DP (1976) Pathways of microbial metabolism of parathion. *Appl Environ Microbiol* 31:63–69
- Murray KC, Duggleby J, Sala-Trepal JM, Williams PA (1972) The metabolism of benzoate and methylbenzoates via the *meta*-cleavage pathway by *Pseudomonas arvilla* mt-2. *Eur J Biochem* 28:301–310

- Prakash D, Chauhan A, Jain RK (1996) Plasmid-encoded degradation of *p*-nitrophenol by *Pseudomonas cepacia*. *Biochem Biophys Res Commun* 224:375–381
- Ramanathan MP, Lalithakumari D (1999) Complete mineralization of methylparathion by *Pseudomonas* sp. A3. *Appl Biochem Biotechnol* 80:1–12
- Rani NL, Lalithakumari D (1994) Degradation of methyl parathion by *Pseudomonas putida*. *Can J Microbiol* 40:1000–1006
- Roldan MD, Blasco R, Caballero FJ, Castillo F (1998) Degradation of *p*-nitrophenol by the phototrophic bacterium *Rhodobacter capsulatus*. *Arch Microbiol* 169:36–42
- Serdar CM, Murdock DC, Rohde MF (1989) Parathion hydrolase gene from *Pseudomonas diminuta* MG: subcloning, complete nucleotide sequence, and expression of the mature portion of the enzyme in *Escherichia coli*. *Biotechnology* 7:1151–1155
- Siddaramappa R, Rajaram KP, Sethunathan N (1973) Degradation of parathion by bacteria isolated from flooded soil. *Appl Microbiol* 26:846–849
- Singh BK, Walker A (2006) Microbial degradation of organophosphorus compounds. *FEMS Microbiol Rev* 30:428–471
- Somara S, Siddavattam D (1995) Plasmid mediated organophosphate pesticide degradation by *Flavobacterium balustinum*. *Biochem Mol Biol Int* 36:627–631
- Spain JC, Gibson DT (1991) Pathway for biodegradation of *p*-nitrophenol in a *Moraxella* sp. *Appl Environ Microbiol* 57:812–819
- Takeo M, Yasukawa T, Abe Y, Nihara S, Marda Y, Negoro S (2003) Cloning and characterization of a 4-nitrophenol hydroxylase gene cluster from *Rhodococcus* sp. PN1. *J Biosci Bioeng* 95:139–145
- Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG (1997) The ClustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res* 24:4876–4882
- Weigent DA, Nester EW (1976) Purification and properties of two aromatic aminotransferases in *Bacillus subtilis*. *J Biol Chem* 251:6974–6980
- Zhang R, Cui Z, Jaingdong J, Jain H, Xiangyang G, Sphunpeng L (2005) Diversity of organophosphorus pesticide degrading bacteria in a polluted soil and conservation of their organophosphorus hydrolase genes. *Can J Microbiol* 51:337–343