Biodegradation of carbaryl and phthalate isomers by soil microorganisms

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Abstract

Pseudomonas sp. strain PP4 and C5 utilize phthalate isomers (*o-, m-* and *p-*) and carbaryl as carbon source, respectively. Degradative pathways were elucidated by isolating and characterizing metabolites, whole-cell O_2 uptake and enzyme activity studies. Metabolic studies suggest that phthalate isomer degrading pathways converge at 3,4-dihydroxybenzoic acid. Phthalate dioxygenases responsible for the degradation of the respective phthalate isomers are induced specifically, suggesting that probably there are three different phthalate dioxygenases. This was supported by whole-cell O_2 uptake studies and cells grown on glucose failed to show the activity of phthalate pathway enzymes. Glucose-grown cells lost the phthalate degradation property indicating probable involvement of plasmid, which is expressed and maintained selectively in the presence of phthalate isomers. The metabolic studies with *Pseudomonas* sp. strain C5 suggest that carbaryl is first hydrolyzed to 1-naphthol by carbaryl hydrolase. Generated 1-naphthol is metabolized *via* 1,2-dihydroxynaphahtlane, salicylate and gentisic acid to TCA cycle intermediates, thus serving as the sole source of carbon and energy. The ability to utilize phthalates (0.3%) and carbaryl (1%) at high concentrations make these strains suitable candidates for bioremediation. Detailed understanding of metabolic pathways and genetic make-up will enable one to modify these strains by genetic engineering tools for suitable application in bioremediation.
Keywords: Phthalate degradation, Carbaryl metabolism, oxygenases, bioremediation, pseudomonas.

1 Introduction

Aromatic hydrocarbons are used heavily in various industries, which found their way in to soil, water and air thus polluting the environment. Due to the persistence of these toxic and carcinogenic compounds in nature, microbes have evolved with novel metabolic pathways to release the 'Locked Carbon'. Phthalate isomers (*o-, m-* and *p-*) and their esters are major industrial pollutants, used heavily in plastic, textile, paint, pesticide carrier, munition, and cosmetic industries. The majority of them are recalcitrant and toxic causing teratogenic, reproductive and neuromuscular disorders [1]. The microbial metabolism of *o*-phthalate is well studied. Many *Pseudomonas* sp. have been reported to degrade *o*-phthalate and phthalate esters to 4,5-dihydroxyphthalate, which enters into the TCA cycle *via* 3,4-dihydroxybenzoate [2]. However very little is known about the degradation of tere- and iso-phthalate. So far there are no reports on a single bacterial strain degrading all three phthalate isomers as the sole carbon source. Besides an environmental pollutant, isophthalate is known to be a competitive inhibitor of glutamate dehydrogenase (GDH) responsible for conversion of α-ketoglutarate to glutamate [3].

Carbamate insecticides, such as carbaryl (1-naphthyl-*N*-methylcarbamate), are highly toxic with a wide range of activities and are used heavily in agricultural industry. Carbamates are competitive inhibitors of neuronal nicotinic acetylcholine receptors and acetylcholinesterase [4]. *N*-Nitrosocarbamates and 1-naphthol generated are potent mutagens, more toxic and recalcitrant than carbaryl itself [5, 6]. Microbial metabolic studies have indicated that the first step in degradation is hydrolysis of carbaryl to 1-naphthol by hydrolase. Depending on the strain, 1-naphthol is metabolized *via* salicylate to either gentisate or catechol [7-10], however the steps and enzymes responsible for the conversion of 1-naphthol to salicylate have not been demonstrated so far.

We have isolated two soil bacterial strains capable of utilizing phthalate isomers and carbaryl. Here we are presenting the metabolic pathway and inhibition of GDH by isophthalate in strain PP4. Based on the metabolic studies, we propose the carbaryl degradation *via* 1,2-dihydroxynaphthalene (1,2-DHN) in *Pseudomonas* sp. strain C5.

2 Materials and methods

2.1 Organism and growth

Pseudomonas sp. strain PP4 and C5 were isolated by an enrichment culture technique from soil contaminated with petroleum products. Strains were grown on Minimal Salt Medium [11] at 30°C with appropriate carbon source.

2.2 Metabolite isolation, bio-transformation and whole-cell O_2 uptake studies

To isolate and identify carbaryl metabolites, a late-log phase spent medium was acidified to pH 2 with 2N HCl and extracted with equal volume of ethyl acetate,

dried over anhydrous sodium sulfate, concentrated and analyzed by TLC using Hexane:Chloroform:Acetic acid 8:2:1 (v/v/v). Metabolites were identified by TLC (R_f and UV-fluorescence), GC-MS and UV-Visible spectroscopy. GC-MS analysis was carried out on Hewlett-Packard G1800A mass spectrometer attached to a gas chromatograph as described [12]. Whole-cell O_2 uptake rates were monitored as described [13], using an Oxygraph (Hansatech, UK) fitted with Clark's type O_2 electrode. Rates were corrected for endogenous O_2 consumption and expressed as nmol of O_2 consumed min^{-1} (mg wet cells)$^{-1}$.

2.3 Preparation of cell-free extracts, enzyme assays and activity staining

PP4 cells were harvested, washed and suspended in buffer HEPES (20 mM, pH 8.5), EDTA (1 mM) and NaCl (100 mM) and disrupted by sonication using an Ultrasonic processor (model GE130, USA) at 4ºC. The cell homogenate was centrifuged and the supernatant was used as the source of enzyme. GDH was assayed spectrophotometrically and activity staining was performed as described [14]. Pseudomonas C5 cells were suspended in K-phosphate buffer (50 mM, pH 7.5, K-PO_4 buffer) and disrupted by sonication at 4ºC, 4 cycles of 15 pulses each, out-put 11 watt. Cell homogenate was centrifuged at 40,000 g for 30 min at 4ºC. The clear membrane-free supernatant obtained was referred to as cell-free extract and used as enzyme source. Carbaryl hydrolase (CH) was monitored spectrophotometrically by measuring the rate of increase in absorbance at 322 nm due to formation of 1-naphthol. The reaction mixture (1 ml) contained substrate (100 µM), an appropriate amount of enzyme and K-PO_4 buffer. The activity was calculated by using molar extinction coefficient of 1-naphthol ε_{322nm} 2200 in K-PO_4 buffer. 1-Naphthol hydroxylase (1-NH) was monitored by the oxygraph method. The reaction mixture (2 ml) contained K-PO_4 buffer, substrate (100 µM), FAD (6.25 µM) and NADH (100 µM). Reaction was started by addition of an appropriate amount of enzyme. 1,2-Dihydroxynaphthalene dioxygenase (12DHNO, 15), gentisate dioxygenase (GDO, 16), protocatechuate dioxygenase (PDO, 17) and catechol dioxygenase (CO, 18) were monitored as described. The specific activities are reported as nmol.min^{-1}.mg^{-1} of protein. Protein estimation was carried out as described [19].

3 Results and discussion

3.1 Metabolism of phthalate isomers by Pseudomonas strain PP4

*Pseudomonas s*train PP4 has the ability to utilize all three-phthalate isomers as the sole source of carbon. Cell respiration studies are summarized in Table 1. Cells grown on isophthalate showed good respiration on isophthalate and 3,4-DHB and similar results were observed for other phthalate isomers. The cells failed to respire on 2,3-DHB and 2,5-DHB (Table 1). These results suggest that specific phthalate isomer induces respective phthalate dioxygenase responsible for the initial ring hydroxylation, which is further metabolized to TCA cycle intermediate *via* 3,4-DHB. Glucose-grown cells failed to show O_2 uptake

suggesting that the enzymes are inducible. Based on these results the proposed pathway is as shown in Fig.1.

Table 1: Whole-cell O_2 uptake rates by strain PP4 grown on different carbon sources (Iso, Isophthalate; Pht, Phtalate; Tere, Terephthalate; Glc, Glucose; DHB, dihydroxybenzoate; nd, not detected; tr, O_2 uptake < 0.5 nmol min^{-1} mg^{-1}).

	Cell respiration on intermediates, nmol O_2 consumed min^{-1} mg^{-1}					
Carbon	Iso	Pht	Tere	3,4-DHB	2,3-DHB	2,5-DHB
Iso	1.6	nd	nd	2.6	tr	tr
Pht	nd	3.5	nd	2.7	tr	1.2
Tere	nd	nd	3.5	1.9	nd	tr
Glc	tr	tr	tr	tr	tr	tr

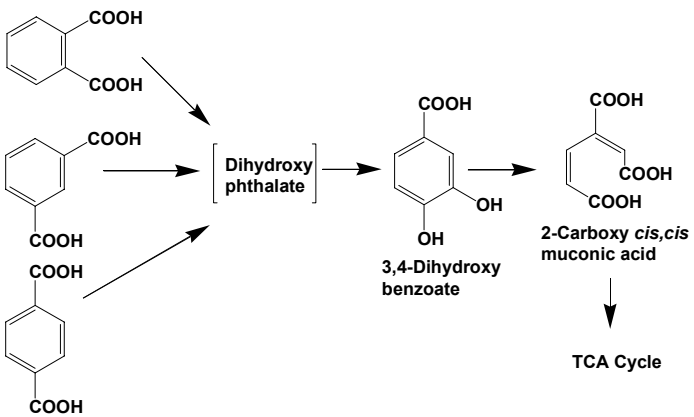

Figure 1: Proposed pathway for the degradation of three phthalate isomers by strain PP4.

The metabolic pathway for phthalate is studied in detail for various enzymes involved, gene regulation and role of plasmids. However, not much information is available on the degradation of iso- and tere-phthalates. Strain PP4 is unique and utilizes all phthalate isomers and converges metabolic pathway into 3,4-DHB. Generated 3,4DHB is ring cleaved to 2-carboxy-*cis,cis*-muconic acid which enters TCA cycle, thus serving as the sole source of carbon and energy. When grown on simple carbon source like glucose, the cells lost their ability to degrade phthalate isomers, suggesting that probably the degradation phenotype is unstable in the absence of specific carbon source and probably genes are located on the plasmid.

3.2 Isophthalate as GDH inhibitor

GDH catalyzes reversible reaction, and equilibrium favors glutamate synthesis. In microorganisms, the biosynthetic reaction of GDH (ammonia assimilating) is catalyzed by NADP-GDH while the oxidative deamination reaction is catalyzed by NAD-GDH. Strain PP4 has NADP-GDH when grown on isophthalate. Glutamate-grown cells had high NAD-GDH activity compared to glucose-grown cells (Table 2). Inhibition of GDH by isophthalate (1mM) is shown in Table 2. GDH from glucose grown PP4 is more sensitive (66%) to isophthalate inhibition compared to GDH from isophthalate grown cells (28%).

Table 2: Specific activity of GDH and its inhibition by isophthalate (1 mM). (Glc, glucose; Glu, glutamate; nd, no activity detected; values in bracket are % inhibition).

Growth on	Amination reaction		
	NADH	NADPH	NADPH+Iso
Iso	nd	231	166 (28)
Glc	28	184	63 (66)
Glu	108	41	22 (47)

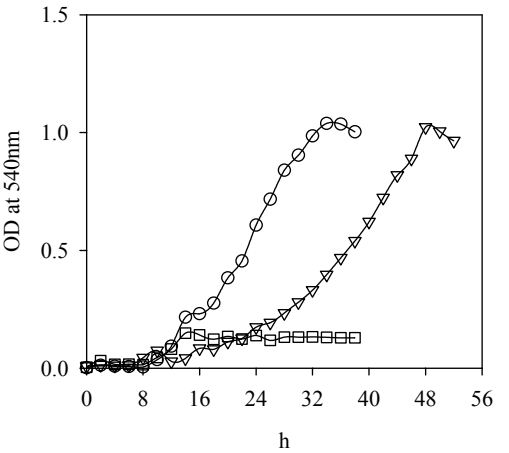

Figure 2: Effect of glutamate on the growth profile of strain PP4 on isophthalate (▽); iosphthalate + glutamate (○) and glutamate (□).

Strain PP4 when grown on isophthalate showed a lag phase of ~ 20 h and reached a stationary phase by 48 h. When supplemented with 1mM glutamate, cells grew faster with short lag phase (12 h) and reached a stationary phase around 34 h (Fig. 2). In the initial phase of the growth, glutamate is taken up by the cells, which might be helping to overcome the inhibition by isophthalate and hence the culture is growing faster with small lag phase. NADP-GDH activity

staining was done with crude extracts from PP4 grown on different carbon sources. The staining pattern for PP4 on isophthalate showed a staining band of lower mobility compared to glucose grown cells. These results suggest that in strain PP4 two GDH isozymes are present and induced depending on the carbon source.

Rapid growth on isophthalate in the presence of glutamate and carbon source dependent changes in the isozyme pattern suggests inhibition of GDH by isophthalate *in vivo*. Probably, isophthalate is acting as a metabolic inhibitor.

3.3 Metabolism of Carbaryl by *Pseudomonas* sp. strain C5

To elucidate the carbaryl degradative pathway, metabolites were extracted and resolved by TLC. Four major spots with R_f, UV fluorescence and GCMS properties similar to authentic carbaryl, 1-naphthol, salicylate and gentisate were identified (Table 3). The analysis failed to detect spots corresponding to 1,4-naphthoquinone and catechol. To elucidate the metabolic sequence, biotransformation experiments were performed using carbaryl, 1,4-naphthoquinone and salicylaldehyde. When cells were supplemented with carbaryl, TLC showed metabolite spots similar to authentic carbaryl, 1-naphthol, salicylate and gentisate. With salicylaldehyde, two major metabolites salicylate and gentisate were detected. However no metabolites with 1,4-naphthoquinone were observed.

Table 3: Identification of carbaryl metabolites from the spent media.

TLC		UV-Vis (nm)	MS analysis {m/z (% relative intensity) [molecular ion]}	Inference
R_f	UV-Fluo			
0.62	Dark blue	280, 312, 318	201(4)[M^+], 144(100), 127(3), 115(52), 89(10), 77(2)	Carbaryl
0.73	Brown black	298, 308, 323	144(100)[M^+], 115(77), 88(10), 77(2)	1-Naphthol
0.77	Sky blue	306	--	Salicylate
0.15	Blue-green	338	154(37)[M^+], 136(100), 108(10), 77(1)	Gentisate

To elucidate steps involved in the conversion of 1-naphthol to salicylaldehyde, whole-cell O_2 uptake rates, enzyme activities in the cell-free extracts and products of bulk enzyme reactions were monitored. Cells showed good O_2 uptake on carbaryl, 1-naphthol, 1,2-DHN, salicylate and gentisate (Table 4). Salicylate grown cells showed O_2 uptake on salicylate and gentisate but significantly low respiration on carbaryl, 1-naphthol, 1,2-DHN and salicylaldehyde. However, glucose grown cells showed very low respiration (Table 4).

Table 4: Whole-cell O_2 uptake rates for cells grown on various carbon sources.

	O_2 uptake* (nmol of O_2 consumed $min^{-1}.mg^{-1}$ cells), cells grown on		
	Carbaryl	Salicylate	Glucose
Carbaryl	3.8	tr	tr
1-Naphthol	7.8	tr	tr
1,2-DHN	1.0	tr	tr
1,4-Naphthoquinone	tr#	tr	tr
Salicylaldehyde	4.4	0.4	tr
Salicylate	1.2	0.7	tr
Gentisic acid	1.7	1.0	tr
Catechol	tr	tr	tr

* Values corrected for endogenous O_2 uptake; # tr, uptake rates < 0.3 nmol.

Table 5: Various enzyme activities from the cell-free.

Enzyme	Specific activity, $nmol.min^{-1}.mg^{-1}$ protein		
	Carbaryl	Salicylate	Glucose
CH	71	1.1	1.1
1-NH	270	4	2
1,2-DHNO	52	32	29
GDO	425	601	1
CO	56	55	39

Specific activities of CH, 1-NH, 1,2-DHNO and GDO are summarized in Table 5. Salicylate cultures showed a comparable activity of GDO and a significantly low activity of CH and 1-NH. All enzyme activities from glucose grown cells were significantly low (Table 5). The enzyme activities and whole-cell O_2 uptake rates from carbaryl and glucose grown cells suggest that the enzymes are inducible. Involvement of 1-naphthol and salicylate in carbaryl metabolism is reported earlier. Here we demonstrate, for the first time, the presence of 1-NH and 1,2-DHNO activities in the cell-free extract of carbaryl degrading strains. 1-NH was studied for its cofactor and O_2 requirement. Under aerobic conditions the cell-free extract showed conversion of 1-naphthol to salicylate in the presence of NADH and FAD. However under anaerobic conditions, we failed to detect salicylate. These results suggest that the enzyme is oxygenase type and requires O_2 for its optimum activity. The enzyme showed good activity with FAD and NADH. When NADPH and FAD were used as cofactors, 20-30% increase in activity was observed.

Based on metabolite studies, we propose a degradative pathway for carbaryl in strain C5 as shown in Fig. 3.

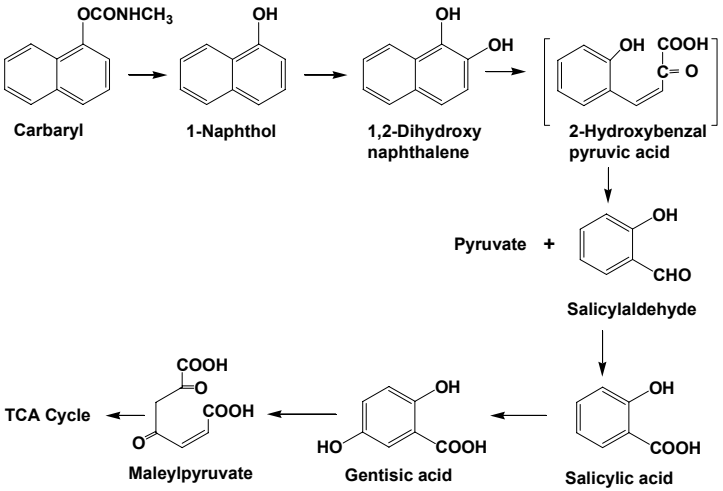

Figure 3: Proposed pathway for the degradation of carbaryl in *Pseudomonas* sp. strain C5.

It has been proposed that prior to ring cleavage, 1-naphthol is hydroxylated either to 4-hydroxy-1-tetralone [21], 3,4-dihydro-dihydroxy-1(2H)-naphthalenone [22] or 1,4-naphthoquinone [23]. Detection of 1-NH, and 1,2-DHNO activity and whole cell O_2 uptake on 1-naphthol and 1,2-DHN strongly suggests 1,2-DHN as intermediate in the pathway. The pathway from 1,2-DHN onward is similar to the naphthalene metabolic pathway, however strain C5 failed to utilize naphthalene.

In conclusion, strains PP4 and C5 are unique, degrade phthalate isomers and carbaryl at higher concentrations, respectively. These strains are isolated from the environment and are very efficient in degradation of compounds of interest. Further characterization and genetic engineering of these strains helps in the degradation of a wide range of aromatic hydrocarbons efficiently. Development of such a metabolically engineered strain has an advantage and will help in effective bioremediation and environmental pollution clean up processes.

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