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Growth of *Pseudomonas aeruginosa* LP5 on 2, 5-dichlorobenzoate: Detection of aromatic ring hydroxylating dioxygenase (ARHDO) gene

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***Pseudomonas aeruginosa* LP5 grew on 2, 5-dichlorobenzoate with doubling time (D) 6.64 d and mean growth rate (k) 0.104 d^{-1}. The organism showed a prolonged lag period lasting 9 days followed by a sudden rise within 3 days (D=1.1 d; k =0.628 d^{-1}) and death in less than 72 hours on 2,6-dichlorobenzoate. Polymerase chain reaction (PCR) amplification of DNA of LP5 showed aromatic dihydroxylating (ARHDO) gene band with molecular weight corresponding to the targeted fragment (0.73 kb). The capability of LP5 on dichlorobenzoates and detection of dioxygenase genes is a validation of its versatility and potential for bioremediation.**

Keywords: Biodegradation, dioxygenase, *Pseudomonas*.

INTRODUCTION

Chlorobenzoates (CBAs) are monoaromatic compounds with chlorine substituents on the benzene ring. They are found in the environment as highly recalcitrant metabolites of degradation of polychlorinated biphenyls (PBCs) and chlorobenzenes (Barton and Crawford, 1988; Kobayashi et al., 1996; Adebusoye et al., 2008). They are also present as components of pesticides and xenobiotics from industrial and agricultural wastes. Organisms that use chlorobenzoates, including 2, 5-dichlorobenzoate, have been well studied (Bedard and Haber 1990; Kozlovsky *et al.*, 1993). However, microbial degraders of chlorobenzoates are not very common in the environment and even when isolated by enrichment or in microcosms, long periods of acclimation are often required for degradation in pure culture (Knackmuss, 1984; Hernandez et al., 1995). Indeed, the majority of reports of aerobic chlorobenzoate utilizers have been on degraders of monochlorobenzoic acids (Hichey and Sabat, 2001; Urgun-Demirtas et al. 2006; Zhang et al. 2010), while fewer reports of dichlorobenzoate and higher chlorinated benzoates are available.

The first step in the aerobic metabolism of aromatic hydrocarbon compounds usually involves the incorporation of the two atoms of oxygen molecule into the benzene ring to form a dihydrodiol which is further stoichiometrically converted to catechol (Blumer, 1976; Cerniglia, 1992; Kim and Zylatra, 1999; Kanaly and Harayama, 2000; Kim et al., 2005). This process destroys the aromatic system and prepares the ring for cleavage (Butler and Mason, 1997; Boyd and Sheldrake, 1998). In many chlorobenzoates this usually proceeds through chlorocatechol (Hickey and Focht, 1990; Sylvestre and Sondossi, 1994). It is catalysed by ring hydroxylating dioxygenases, which are a family of multicomponent enzymes consisting of a hydrolase and an electron transport system. The dioxygenase genes encoding the enzymes have been cloned in a number of aromatic hydrocarbon-degrading bacteria (Resnick et al., 1996; Khan et al., 2001; Krivobok et al., 2003; Kim et al., 2006). Kahl and Hofer (2003) designed primers for the amplification of this core region, and it was shown that this system could be used to rapidly isolate and functionally characterize aromatic ring-hydroxylating properties from isolated strains, mixture of organisms or samples of nucleic acid. We had documented in an earlier report the ability of strain LP5 to utilise pyrene as sole carbon and energy source and also showed that ring cleavage of catechol proceeded by ortho pathway

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(Obayori et al., 2008). Here we report the ability of our isolate to grow on 2, 5-dichlorobenzoate and the ARHDO gene.

MATERIALS AND METHODS

Microorganism and inoculum development

The organism used in this study, *Pseudomonas aeruginosa* strain LP5, was isolated on the basis of its ability to utilise pyrene as sole sources of carbon and energy as previously described (Obayori et al., 2008). The organism was maintained in glycerol: nutrient broth (1:1) at -20°C. Colonies growing on Luria-Bertani agar were harvested with sterile inoculating loop, pooled and transferred to screw-capped bottles containing 5 ml of physiological saline (0.9% NaCl). Enough cells were transferred to achieve an OD_{600} of approximately 1.5.

Medium and culture conditions

The mineral salts medium (MSM) described by Kastner et al. (1994) in which the chloride salt had been substituted with sulphate was used. The medium contained per liter.: Na_2HPO_4, 2.13 g; KH_2PO_4, 1.30 g; NH_4SO_4, 0.50 g and $MgSO_4$. $7H_2O$, 0.20 g. The pH of the medium for bacteria was adjusted to 7.2 and further fortified with trace elements solution (1 ml/l) previously described by Bauchop and Elsden (1960). The medium was supplemented with 0.005 % yeast extract. The trace elements solution was sterilized separately and added aseptically to the medium.

Growth experiment

Replicate flasks containing 50 ml of MSM with 100 ppm of chlorobenzoates were prepared. Isolate was inoculated to achieve an initial concentration of the total viable count (TVC) of about 1.0×10^6 cfu/ml. Total viable counts and pH were determined at 3 days interval by plating out appropriate dilutions of the cultures onto nutrient agar. Incubation was carried under aerobic condition (150 rpm) at room temperature (27 ± 2.0 °C). Growth data were subjected to non-linear regression analysis using the Prism version 5.03 computer software programme (GraphPad Software, San Diego, CA. USA). Significance limits were set at the 95% probability level

Detection of dioxygenase gene by Polymerase Chain Reaction (PCR)

Cell lysis was carried out by suspending small portion of

colony in 200 µl of distilled water in a 1.5 ml eppendorf tube and boiling for 10 min. The lysate was used immediately or preserved by freezing. Amplification of DNA was carried out in thermal cycler (Eppendorf Mastercycler, Model 5332, Hamburg, Germany).

The forward primer (HDO2AF) was 5'–AGGCGGAGACCTACAAGGGCCTGATTTTCGCCAACTGGGA-3' and the reverse primer (HDO2AR),GCCTTGTGGCCGCTGCTGGATCTCGACCCAGTTCTCGCCGTCGTCCTG (Kahl and Hofer, 2003).The reaction mixture consisted of 5 µl of BIO-X-ACT short PCR MIX (Bioline, USA), 0.2 µl each of the forward and reverse primers, 0.5 µl of cell lysate and 4.1 µl of distilled water in an eppendorf tube (200 µl). The initial denaturation temperature was 94°C for 3 min, followed by 40 cycles of denaturation at 94 °C for 1 min, annealing at 50 °C for 1 min and elongation at 72 °C for 1 min. The final elongation was at 72 °C for 7 min. It was then cooled to 4 °C.

For the detection of PCR product, electrophoresis was carried out on agarose gel (0.7%) at 100 V- 200 mA (Consort 300 V-500 mA, E835, Electrophoresis Power Supply, Belgium) for 1 h after loading entire mix into well with 1 µl loading buffer. The marker was Ladder (0.5 - 10 kb) (New England Biolabs, USA). Gel was observed under UV light after staining with ethidium bromide (5 mg/l) for 15 min.

RESULTS AND DISCUSSION

Growth of isolate on chlorobenzoates

The time course of growth of *Pseudomonas aeruginosa* LP5 on 2, 5-dichlorobenzoate is shown in Figure 1. The organism exhibited a steady increase from population density of 9.0×10^6 at the onset of incubation to 1.64×10^8 on Day 18 and then declined to 1.58×10^8 Day 21. The doubling time (D) was 6.64 and mean growth rate (k) 0.104 d^{-1}. The pH of the medium fluctuated between 6.64 and 7.14. The steady growth exhibited by LP5 on 2, 5- dichlorobenzoate suggests that this organism did not produce dead-end product(s) and actually metabolised the substrate to biomass. The versatility of LP5 on chlorobenzoate may not be unconnected with the fact that it was originally isolated from a site prone to contamination with complex mixtures of hydrocarbons including chlorinated compounds (Obayori et al. 2008).

P. aeruginosa LP5 also grew on 2-chlorobenzoate, and 2,6 dichlorobenzoate but not on 3- and 4- chlorobenzoate. This is not too different from earlier reports which showed that strains of *Pseudomonas* species could utilise mono and dichlorobenzoates (Ghosal et al., 1985; Hickey et al., 1993). Similarly, Kozlovsky et al. (1993) showed that a *Pseudomonas stutzeri* strain KS25 utilized 2-chlorobenzoic and 2,5-dichlorobenzoic acids but not 3-, 4- chloro and 2,6-
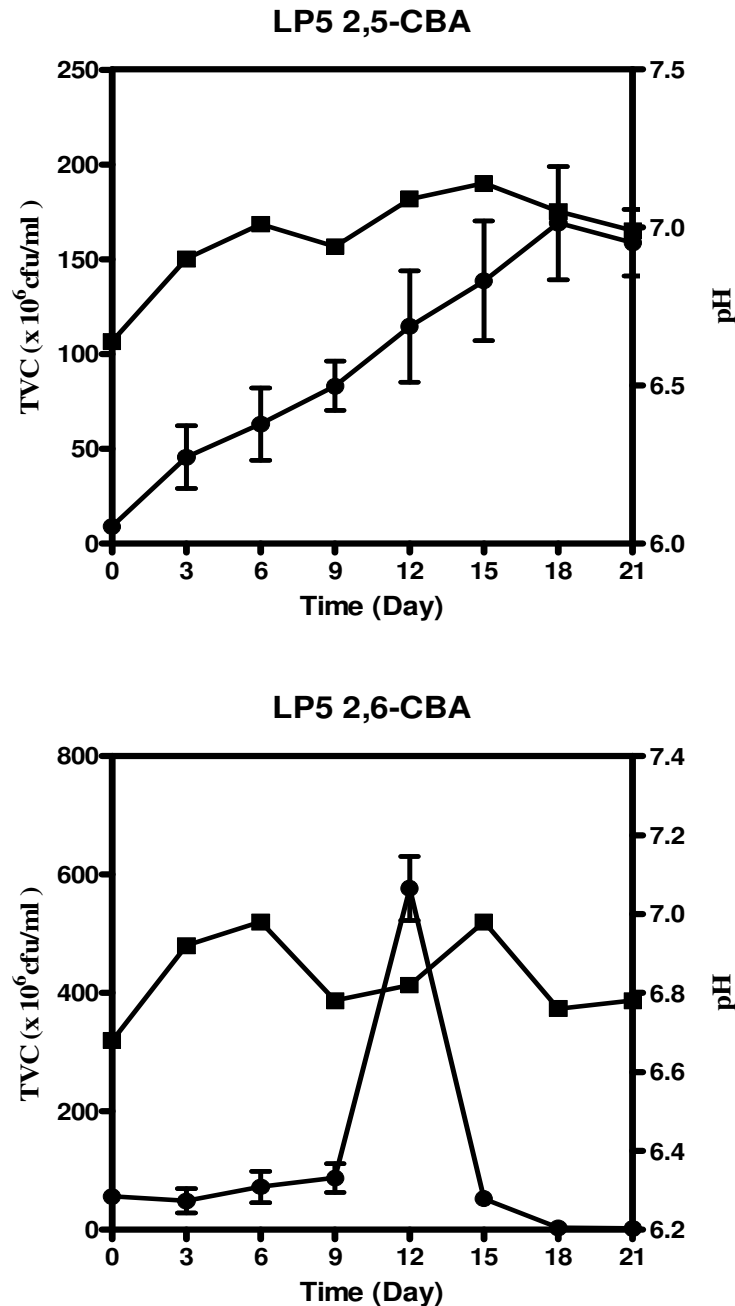
LP5 2,5-CBA

LP5 2,6-CBA

Figure 1 Time course of growth of *Pseudomonas aeruginosa* strain LP5 on 2,5-dichlorobenzoates (up) and 2,6-dichlorobenzoate (down) showing growth (●) and pH changes (■). Plots are means of replicate results. Incubation was carried out under aerobic condition (150 rpm) at room temperature (27 ± 2.0 °C).

dichlorobenzoate. The organism (LP5) showed pro-longed lag period lasting 9 days followed by a sudden rise to 5.5×10^8 cfu/ml on Day 12 (D= 1.1 d; k =0.628 d^{-1}) and death in less than 72 hours and non-detection of any cell at all six days post-peak when supplied with 2,6-chlorobenzoate as sole carbon and energy sources.

This pattern of growth suggests that the organism may have produced toxic metabolites in the medium leading to eventual death.

Recently, Adebusoye and Miletto (2010) showed that a large number of mono- and dichlorobenzoic acid degraders spanning 17 genera were important members
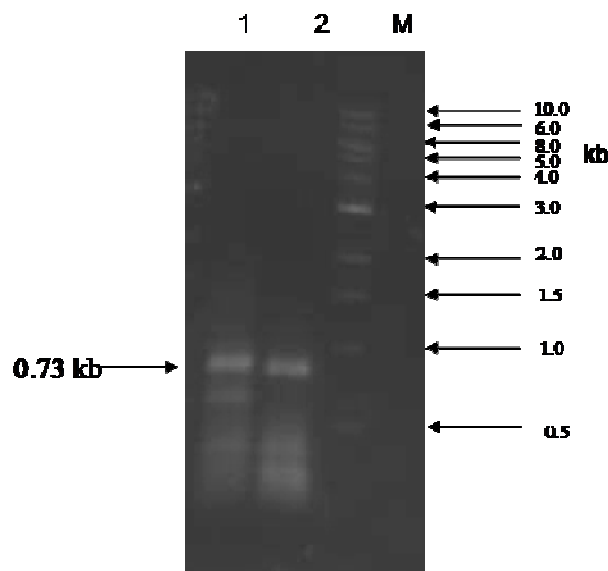
Figure 2. Ethidium bromide-stained agarose gel (0.7%) electrophoresis of PCR products (annealing temp., 50°C) of hydroxylating gene DNA fragments of isolates.   Lane 1, *P. aeruginosa* strain LP5; lane 2, *P. aeruginosa* strain LP6; M, molecular marker, 1 kb DNA ladder.

of the microbial community of contaminated sediments, water and soils. The organisms from soil were found to be strains of the genus Burkholderia, while the water enrichment yielded strains closely related to *Alteromonas, Marinomonas, Lysinibacillus* and *Bacillus.* The sediment community was even found to be more diverse. This suggests that a wide vartiety of organisms than earlier thought may be capable of processing chlorobenzoates.

Detection of Aromatic Ring Hydroxylating Dioxygenase Genes

DNA amplification of ring hydroxylating dioxygenase genes in LP5 was carried out at annealing temperature of 50°C (Figure 2). Result shows aromatic dihydroxylating gene bands with molecular weight corresponding to the targeted fragment (730 bp). This result shows that the organisms harbour ring-hydroxylating genes that may be similar to *Burkolderia* species strain LB 400. The primers were made from conserved regions and were found to be good for some pseudomonads. Similar gene was amplified in *Pseudomonas* sp. B2A, *Ralstonia* sp. H850 and *Rhodococcus globerulus* P6 among others (Kahl and Hofer, 2003) which are prolific PCB degraders. Since PAHs and PCBs have benzene as the units of their structures, it is therefore not surprising that primers primarily developed to amplify aromatic ring hydroxylating dioxygenases in PCB degraders will also

amplify similar genes in PAH degraders and the same isolate equally hydroxylated chlorinated aromatic rings. Furthermore, enzymes coded for by the genes are similar, yielding *cis,cis*-dihydrodiols compounds from both PCBs and PAHs from which the primer was developed. This result is also a further validation of our enzyme study result (Obayori et al., 2008) which showed these organisms to possess aromatic ring cleavage activity, since hydroxylation is a precondition for aerobic ring cleavage (Cerniglia, 1992). The ability of *Pseudomonas aeruginosa* strain LP5 to utilize 2,5-diclorobenzoate suggests that this organism has other genes encoding the enzymes for processing this substrate in its gene pool. Significantly, it has been posited that being readily available as a result of their high solubility in the aqueous phase, lack of presence of gene in the gene pool rather than low bioavailability accounts for poor degradability of chlorobenzoates (Adebusoye and Milleto, 2010).

Previously we had demonstrated the versatility of LP5 on a pyrene, dibenzothiophene and petroleum cuts such as diesel, engine oil and kerosene (Obayori et al. 2008). The capabilities of LP5 on dichlorobenzoates and detection of dioxygenase genes as demonstrated in this study represents further validation of its versatility and potential for bioremediation.

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