Degradation of *p*-hydroxybenzoate via protocatechuate in *Arthrobacter protophormiae* RKJ100 and *Burkholderia cepacia* RKJ200

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The use of microorganisms is expected to be an effective tool for remediation of environments contaminated with various aromatic compounds. Aromatic compounds are degraded via diverse ring-cleavage pathways by soil microorganisms. Two bacterial strains isolated from pesticide-contaminated agricultural fields and identified as *Arthrobacter protophormiae* RKJ100 and *Burkholderia cepacia* RKJ200 were found to be capable of utilizing *p*-hydroxybenzoate (PHB) as a sole source of carbon and energy. Thin layer chromatography, gas chromatography and gas chromatography–mass spectrometry of the ethyl acetate extracts from the culture medium showed the presence of protocatechuate. Both the strains follow the *ortho*-cleavage pathway for the catabolism of PC, since it was found that the terminal aromatic intermediate of the degradation pathway is cleaved to *b*-ketoadipate. Plasmid cured derivatives of *A. protophormiae* and *B. cepacia* were also investigated to determine the location of the genes involved in PHB degradation.

ENVIRONMENTAL pollution results in the uptake and accumulation of toxic chemicals in food chains and drinking water, thereby posing a substantial health hazard for current and future generations. Bioremediation constitutes an attractive alternative to physico-chemical methods of remediation, as it is less expensive and can selectively achieve complete destruction of organic pollutants[1,2]. Simple aromatic compounds and their derivatives can exist in the environment at a higher concentration than desired due to anthropogenic activities and can be a source of environmental pollution. Aromatic compounds are also present as components of complex polymers such as lignin that comprises about 25% of the land-based biomass on earth[3]. Recycling of such aromatic compounds is vital for maintaining the earth's carbon cycle. In recent years, there have been significant efforts toward exploring microbial ability to degrade and detoxify the increasing amounts of aromatic compounds. Despite the vast array of aromatic compounds in aquatic and terrestrial environments, the degradation of different compounds finally involves a limited number of metabolic pathways. An important component of these pathways are the reactions that degrade aromatic rings by the insertion of oxygen directly into the aromatic compound. Understanding the biodegradation of structurally simple, readily degradable aromatic compounds facilitates studies on the more recalcitrant members of the group. *p*-hydroxybenzoate (PHB) is a common intermediate in the microbial degradation of lignin, the principal aromatic component of wood[4–7]. Processing and degradation of aromatic compounds from plant material found in soil, such as those originating from solubilization of lignin occur via the *b*-ketoadipate pathway and it is therefore considered as the 'major utility pathway'[5]. Intermediates formed due to the solubilization of lignin get transformed to PHB and are subsequently degraded via the *b*-ketoadipate or the *ortho* pathway, wherein PHB is converted to 3,4-dihydroxybenzoate or protocatechuate (PC), which is catabolized by the action of PC 3,4-dioxygenase[4,5,8–10]. The ring cleavage product is converted to TCA-cycle intermediates as in the case of *Bradyrhizobium japonicum*[11]. In *Azotobacter chroococcum*, PHB is metabolized via the *ortho* (protocatechuate 3,4-dioxygenase) and *meta* (protocatechuate 4,5-dioxygenase) ring-cleavage pathways[12,13]. In *Rhodopseudomonas palustris*, the degradation of PHB is anaerobic and proceeds via the formation of 4-hydroxybenzoyl CoA[14]. The present article reports the oxidative degradation of PHB by *A. protophormiae* and *B. cepacia* via the formation of PC that is further converted to TCA-cycle intermediates. Furthermore, it was observed that the genes for PHB degradation are plasmid-encoded in *B. cepacia*, whereas they are chromosomally encoded in *A. protophormiae*.

Materials and methods

Bacterial strains

The bacterial isolates *A. protophormiae* RKJ100 and *B. cepacia* RKJ200 were isolated from pesticide-contaminated agricultural fields[15–17]. The strains were grown on 5 mM PHB as the sole source of carbon and energy for studying the growth characteristics of the bacterial strains.

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Growth conditions and preparation of crude cell extract

Minimal medium (MM) used was essentially the same as described earlier[15]. In addition to 5 mM PHB or PC, sodium succinate was also added to the growth medium as carbon source. The overnight cultures (250 ml) of both the strains were induced with either PHB (5 mM) or PC (5 mM) and harvested 1 h after induction. In order to perform enzyme assays, crude cell extract was prepared by washing the overnight-grown cells and resuspending them in 10 ml of chilled assay buffer. Cell lysis was carried out using a chilled French Pressure Cell at 25,000 lb/in^2. The lysates were centrifuged at 21,000 *g* for 30 min at 4°C and the supernatant was used as crude cell extract.

Identification of intermediates of the degradation pathway

For extraction of intermediates of the pathway, following growth of the cultures, they were centrifuged for 15 min at 25°C and the supernatant was extracted with an equal volume of ethyl acetate. The pH of the aqueous layer was lowered to 3.0 by adding HCl and this layer was extracted again with an equal volume of ethyl acetate. The neutral and acidic extracts were pooled and evaporated to dryness in a rotary evaporator in a bath temperature at 40°C. The residue was resuspended in methanol and analysed by various techniques[16]. In order to detect the intermediates formed prior to ring-cleavage, 1 mM 2,2′ dipyridyl was added to the minimal medium containing 5 mM PHB and 20 mM sodium succinate as sources of carbon and energy. After different time intervals, samples were collected, extracted and concentrated as described above.

Colorimetric detection of PC

Formation of PC from PHB was detected colorimetrically as described by Parke[18]. Bacteria were grown to late log phase in MM containing sodium succinate and 5 mM PHB. Further incubation was done at 30°C for 2 h in the presence of $FeCl_3$ (1.5 mM) and *p*-toluidine (50 µg/ml in N,N-dimethylformamide). Appearance of a purple colour is observed PC is produced by the cells growing in the presence of PHB.

Rothera test for the detection of ortho- or meta-cleavage product

Rothera test as described by Holding and Collee[19] with minor changes was performed as follows: 0.2 ml of the whole cell lysate was incubated with the substrate (0.5 mM PC) in 2 ml 0.02 M Tris buffer (pH 8) for 30 min, followed by addition of 1 g ammonium sulphate, 0.02 ml 1% sodium nitroprusside (freshly prepared) and 0.5 ml ammonia solution. Tubes were then incubated for 1 h at 30°C in shaking condition. Appearance of a purple colour indicates *ortho*-cleavage of the substrate.

Analytical techniques

Thin layer chromatography (TLC) was performed with the extracted samples as described above on pre-coated silica gel 60 F_{254} plate (20 cm × 20 cm and 2 mm thick, E. Merck, Germany). The solvent system used was toluene : ethyl acetate : acetic acid (60 : 30 : 5). These samples were also analysed by gas chromatography (GC) (Hewlett Packard 5890 series) during which oven temperature was maintained at 250°C and nitrogen was used as the carrier gas, and the injector and detector were set at 280°C. Authentic standards were used for comparison of results obtained for GC analysis. Gas chromatography–mass spectrometry (GC–MS) analysis was performed using Shimadzu QP 5000 GC–MS, as described earlier[16]. For High Performance Liquid Chromatography (HPLC) studies, samples were withdrawn after different intervals of growth on 5 mM PHB and analysed for the presence of PHB and PC. HPLC was done using a Waters instrument (model 600) equipped with a photodiode array detector operating at a wavelength of 245 nm for the detection of PHB and PC. Separation was carried out with a Waters Spherisorb 5 µm ODS2 column, with an isocratic flow rate of 1 ml/min. The mobile phase consisted of 43% acetonitrile and 57% 15 mM aqueous triethylamine (pH adjusted to 3.9 with *o*-phosphoric acid). The temperature was maintained at 25°C during the entire run.

Enzyme assays

Protocatechuate 3,4-dioxygenase activity was measured spectrophotometrically as described earlier[13,20], with slight modifications, using crude cell extracts. The reaction mixture contained PC (50 µM), Tris-HCl buffer (50 mM, pH 8.5) and crude cell extract (60–100 µg) in a final volume of 1 ml. The reaction was initiated by the addition of substrate and the reaction mixture was scanned from 250 to 450 nm after every 2 min in a spectrophotometer (Perkin Elmer Lambda EZ201 UV/Vis). One unit of enzyme is defined as the amount that oxidizes PC at an initial rate of 1 µM/min. Specific activity was calculated as units per milligram of protein.

The activity of PHB 3-hydroxylase was calculated by the rate of oxidation of NADPH in the reaction mixture containing 0.2 mM PHB, 50 mM NADPH and 50 µM FAD in 50 mM phosphate buffer (pH 7.5)[21]. The addition of FAD was essential for optimal turnover. One unit of enzyme is the amount that catalyses the oxidation of 1 µM of NADPH/min under assay conditions. Enzyme assays were also performed using uninduced crude cell extracts

(cells grown on 20 mM sodium succinate) as negative control.

Isolation of plasmid DNA and mutagenesis

Plasmid DNA isolation was performed by the method of Anderson and McKay[22]. The curing of the plasmids in both strains was carried out using mitomycin C as the curing agent. The cells were grown in increasing concentration of mitomycin C and the minimum inhibitory concentration (MIC) for the curing agent was determined. The cells were grown at sub-lethal levels for 24 h and then transferred to fresh nutrient broth containing mitomycin C at the same concentration. The process was repeated three times. The cells were then plated out on nutrient agar plates and replica-plated on MM agar containing 5 mM PHB. A total of 1000 such colonies randomly selected in each case were tested for their growth on PHB as the sole source of carbon and energy. The colonies that failed to grow on PHB were screened for the presence of plasmid(s). To monitor spontaneous plasmid loss cells were grown in nutrient broth for three cycles as indicated above, but without mitomycin C. Cultures were then diluted and spread on nutrient agar plates. One thousand colonies were picked and replica-plated onto MM agar containing 5 mM PHB to test the loss of the phenotype on PHB.

Chemicals

PHB, PC and 2,2′ dipyridyl were purchased from Sigma Chemical Co, USA. All other chemicals used were of highest purity grade available.

Results and discussion

A. protophormiae and *B. cepacia* were grown on 5 mM PHB and generation time was calculated as 168 min and 192 min respectively. Simultaneous decrease in the concentration of PHB was detected by HPLC analysis as shown in Figure 1 *a* and *b*. Complete degradation of 5 mM PHB occurred in 9 and 8 h in *A. protophormiae* and *B. cepacia* respectively (Figure 2 *a* and *b*). Samples withdrawn from the culture medium after various growth intervals were extracted, concentrated and loaded onto TLC plates along with suitable standards. The results clearly indicated the presence of PHB (Rf 0.52) in samples drawn after 2, 4 and 6 h of growth, along with another compound (Rf 0.28) that could be a possible intermediate of the degradation pathway (Table 1). The rate of migration of this compound on TLC plates was similar to that of standard PC. In samples withdrawn after 12 h of growth, both the compounds could not be detected. Colorimetric detection of intermediates of PHB degradation was also performed and the appearance of a bright purple colour indicated the formation of PC from PHB by both the bacterial strains after overnight growth in the presence of PHB. Samples used for TLC analysis were also analysed by GC in order to determine the intermediate(s) of PHB degradation (Table 1). The results showed the appearance of an intermediate with retention time (Rt) same as that of standard PC. These results were also confirmed by HPLC analysis, where the spectrum of the eluted peak (Rt 2.978 min) was comparable to that of standard PC (Table 1). Most aromatic compounds are first converted to one of several di- or trihydroxylated substrates, whose aromatic ring is further cleaved[8,23]. PHB is mineralized via the formation of PC due to the activity of PHB-3 hydroxylase or via the formation of HQ and BT due to the activity of PHB-1 hydroxylase (decarboxylating enzyme)[24]. In both the strains under study, detection of PHB-3 hydroxylase activity in the crude cell extract confirmed that PHB degradation proceeds via the formation of PC that can be further converted to the intermediates of the *b*-ketoadipate pathway. The activity of this enzyme was found to be 82.5 μM/min in *A. protophormiae* and 98.5 μM/min in *B. cepacia*. Once formed, PC can be cleaved via the *ortho* or

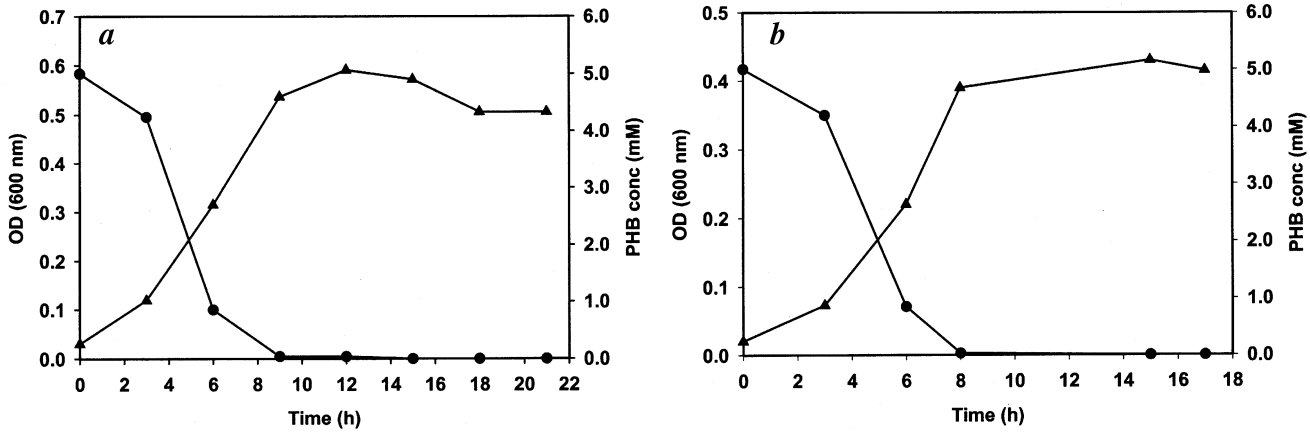

Figure 1. Depletion of PHB by *A. protophormiae* (***a***) and *B. cepacia* (***b***) using HPLC analysis. (●), Depletion of PHB by cells; (▲), Optical density of cells.

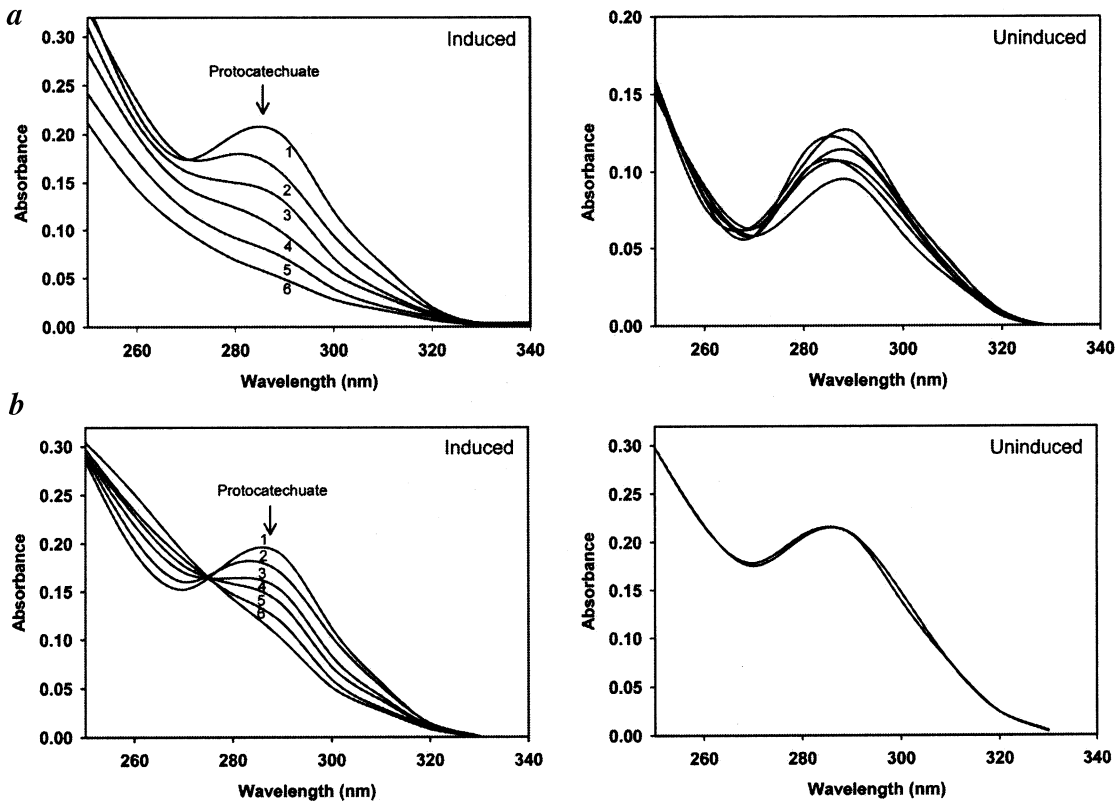

Figure 2. ***a***, Activity of PC 3,4-dioxygenase enzyme in induced and uninduced crude cell extracts of *A. protophormiae*. ***b***, Enzyme activity in induced and uninduced cells of *B. cepacia*.

Table 1. Identification of intermediates formed in the degradation pathway of PHB by *A. protophormiaea* and *B. cepacia* and their mitomycin C-treated derivatives by TLC, GC, and HPLC analyses

	Rf in TLC studies[a]				Rt (min) in GC analysis[b]				Rt (min) in HPLC analysis[c]			
Compound	(s)	(w)	(m_1)	(m_2)	(s)	(w)	(m_1)	(m_2)	(s)	(w)	(m_1)	(m_2)
PHB	0.50	0.52	0.52	0.52	2.658	2.650	2.652	2.652	3.230	3.229	3.232	3.229
PC	0.30	0.28	0.27	–	2.290	2.295	2.299	–	2.972	2.978	2.978	–

[a]Composition of the solvent system is given in the text. Rf values were calculated as distance of analyte from origin to distance travelled by the solvent front from the baseline.

[b,c]GC and HPLC methods are described in the text.

s, Standard compound used; w, Putative compound/intermediate identified in wild-type strains; m_1, Putative compound/intermediate identified in a mitomycin-C-treated derivative of strain RKJ100; m_2, Putative compound/intermediate identified in a mitomycin-C-treated derivative of strain RKJ200.

meta cleavage pathway to form intermediates of the pathway.

To determine whether degradation of PHB via PC followed the *ortho* or *meta* aromatic cleavage pathway, both the organisms were grown in the presence of PHB/PC and the crude cell extract was incubated with PC. The formation of 2-hydroxy-4-carboxymuconic semialdehyde (a yellow metabolite formed due to *meta*-cleavage)[25] was not detected in the reaction mixture in either case, hence proving that the pathway does not follow the *meta* cleavage pathway. The *ortho* cleavage pathway proceeds without the development of any characteristic colour and is catalysed by the enzyme PC 3,4-dioxygenase. Enzyme assays were therefore performed to determine the activity of PC 3,4-dioxygenase in the crude cell extracts of both organisms. The specific activity of the enzyme in the induced cell extracts of *A. protophormiae* and *B. cepacia* was 160 U/mg and 240 U/mg of protein respectively, which confirmed the involvement of the *ortho*-cleavage pathway. Disappearance of PC from the enzymatic reaction was detected by spectrophotomeric assay as PC absorbs at 290 nm (Figure 2 *a* and *b*). Earlier reports have

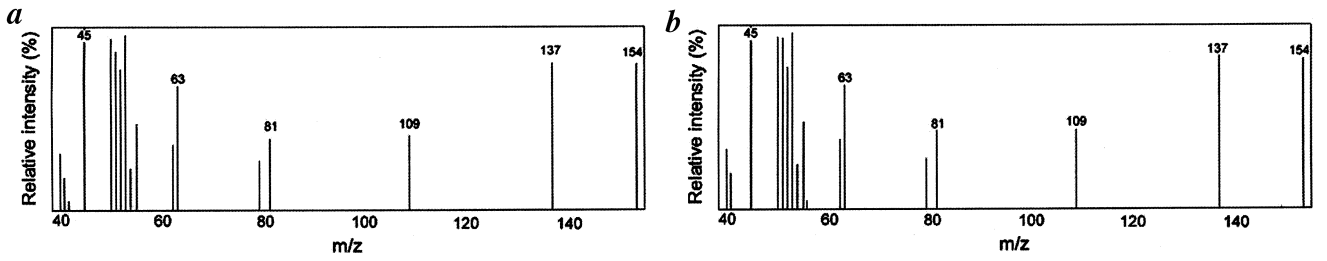

Figure 3. Mass spectrum of PC. ***a***, Authentic standard; ***b***, Present in the culture supernatant of PHB-grown cells of *A. protophormiae* and *B. cepacia*.

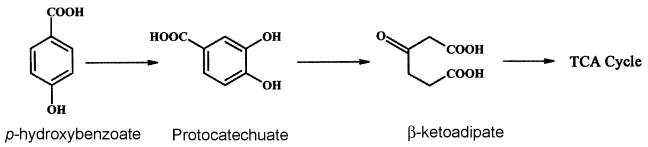

Figure 4. Degradation of PHB via the formation of PC. PC is the substrate for ring cleavage enzymes and the products enter the TCA cycle.

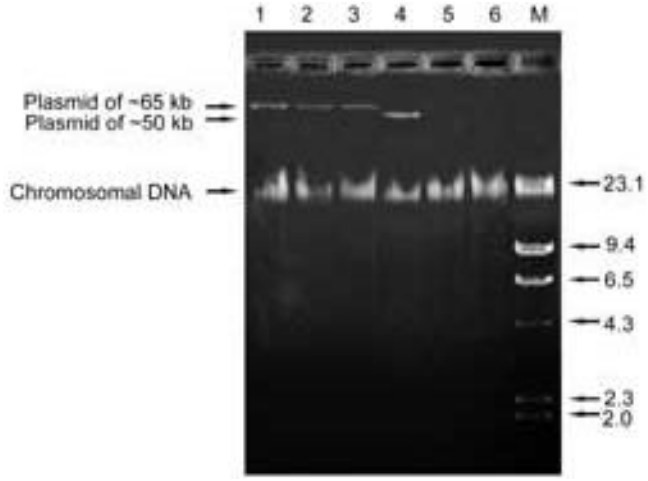

Figure 5. Plasmid profiles of *A. protophormiae* and *B. cepacia* and their mitomycin-C-treated derivatives. Lane 1, *A. protophormiae*; lanes 2–3, Mitomycin-C derivatives of *A. protophormiae*; lane 4, *B. cepacia*; lanes 5–6, Mitomycin-C-treated derivatives of *B. cepacia*; M, Molecular weight marker in kb.

shown that PC is the terminal intermediate of PHB catabolic pathway[8,11,26]. A positive Rothera reaction also indicated that PC was the substrate of ring cleavage in both the organisms.

The above results were further confirmed by addition of 2,2′ dipyridyl to the growth medium, which inhibits ring cleavage and allows the substrate for ring cleavage to accumulate. Samples withdrawn after various growth intervals were used for TLC and GC analyses as before and it was seen that PC accumulated in the growth medium, suggesting that PC is the substrate for ring cleavage. The presence of PC as an intermediate in PHB degradation pathway of these strains was finally confirmed by GC-MS analysis. The mass spectrum of PC accumulated in the culture supernatant of PHB-grown cells of both strains exactly matched with the authentic PC standard (Figure 3). Therefore, it can be concluded that the degradation of PHB in *A. protophormiae* and *B. cepacia* occurred via formation of PC, which was then cleaved to *b*-ketoadipate, an intermediate of the TCA cycle. The pathway is shown in Figure 4. As both the organisms followed the same pathway for PHB degradation, it was of interest to determine the localization of genes for PHB degradation in these organisms. It has been reported earlier that *A. protophormiae* and *B. cepacia* carry plasmids of approximately 65 kb and 50 kb respectively[27]. Attempts were made to obtain PHB^- derivatives to determine the role of plasmid(s) present in the two bacterial strains. It was seen that in *B. cepacia*, the spontaneous loss of PHB^- phenotype was 1% and was increased to 3% when mitomycin C was present during growth. TLC, GC and HPLC analyses of ten such randomly selected PHB^- derivatives showed the absence of PC in the culture supernatant and data for one such derivative have been tabulated (Table 1). When such spontaneously derived or mitomycin-C-treated PHB^- derivatives were checked for the presence of plasmid(s), the ~50 kb plasmid was found to be absent, which indicated its involvement in PHB degradation. Figure 5 shows the plasmid profile of the wild-type strain *B. cepacia* and two of its PHB^- derivatives, which indicated the loss of the ~ 50 kb plasmid. On the other hand, in the case of *A. protophormiae*, no PHB^- derivatives could be obtained either spontaneously or by mitomycin-C treatment. Ten such randomly selected derivatives (obtained after mitomycin C treatment) were screened for PC formation by TLC, GC and HPLC analyses of the culture supernatant. All these derivatives showed the presence of PC in the culture supernatant and data for one such derivative have been tabulated (Table 1). The plasmid DNA isolation study on the above ten PHB^- derivatives selected randomly showed the presence of the ~65 kb plasmid in them. The plasmid profile of two of such derivatives is shown in Figure 5. These results, therefore, suggest that the genes of PHB degradation in *A. protophormiae* could be chromosomally encoded.

PHB is a simple, aromatic compound which is present in soil as a degradation product of lignin. In this study it was found that the strains RKJ100 and RKJ200 could mineralize this compound efficiently. These strains could therefore be useful in studying the mechanism of degradation of complex aromatic compounds and to obtain kinetic models that may assist in the bioremediation of such toxic compounds. Biochemical and genetic analyses of PHB degradation in both the organisms revealed that the strains differed in their growth properties and in the organization of the gene(s) involved in degradation of PHB. It would be interesting to compare the genes of these strains to assess whether they share a common origin or arose independently. It may be possible that strains capable of PHB degradation may also be useful in the bioremediation of soils that are contaminated with related, naturally occurring or xenobiotic aromatic compounds.

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