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Involvement of Plasmid in Degradation of Pentachlorophenol by *Pseudomonas* sp. from a Chemostat

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***Pseudomonas* sp. strain IST103 obtained from a stable bacterial consortium was capable of utilizing pentachlorophenol (PCP) as sole carbon and energy source. The consortium was developed by continuous enrichment in a chemostat. The degradation of PCP by bacterial strain proceeded through an oxidative route as indicated by accumulation of tetrachloro-p-hydroquinone and chlorohydroquinone determined by high performance liquid chromatography (HPLC), and chloride molecules released in culture medium. Two different molecular size plasmids, of approximately 80 and 4 kilobase, were found to be responsible for carrying genes for degradation of PCP. This was evidenced by mutants produced by curing of plasmid by treatment of ethidium bromide. The derivatives were not able to utilize PCP, however, transformation of low molecular size plasmid of *Pseudomonas* sp. strain 103 into *E. coli* JM109 utilized PCP, indicated a possible involvement of plasmid in degradation of pentachlorophenol.** © 2001 Academic Press

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Pentachlorophenol (PCP) is an important organic chemical because of its widespread applications in agriculture, industry, and commercial product formation and preservation (1). It is highly toxic and persists in water and soil for a longer time, and adversely affects flora and fauna (3, 8). PCP degradation by several microorganisms has been reported, but there is disagreement over the reaction mechanisms involved in complete degradation of PCP. Several aerobic bacteria are capable of degrading PCP to tetrachloro-p-hydroquinone (TeCH) by PCP-4-monooxygenase which is further degraded to 2,3,6-trichloro-p-hydroquinone and 2,6-dichloro-p-hydroquinone, and other related compounds (6). But complete degradation of PCP by single bacterial strain has not been reported so far.

Bacterial strains isolated from nature have been proved ineffective for bioremediation of PCP present in industrial effluent because of accumulation of huge amounts of halogenated compounds, position of halogen substituted in the aromatic ring, and disassociation of bacterial strain with the indigenous microflora (7, 15). Furthermore, the genetic analysis has indicated the involvement of genomic DNA of PCP-degradation, but genes are not transferable from one strain to another (13). Nevertheless degrading genes present in plasmid confer great significance in mobility and can be transferred into indigenous population during bioremediation of contaminants at the industrial sites (9). The plasmid may possess the characteristics necessary for growth and survival in the contaminated environment, and thus, establish a stable array of hosts during bioremediation (10, 11). However, there are no reports of involvement of plasmid in degradation of PCP. Therefore, initial goal of the present study is to develop bacterial strain, and to understand molecular mechanism of the strain for degradation so that it can be applied for bioremediation of PCP present in industrial effluent and soil.

MATERIALS AND METHODS

Eighteen sampling sites of three pulp and paper mills located at Lalkua, Bazpur, and Bilaspur (Uttaranchal, India), three sites from main channel of tannery (Jazmau, Kanpur, India), and three sites of S. L. Leather Ltd. (Sirsaganj, Agra, India), were selected. The sediment core together with liquid effluent (1:10 w/v) was collected and stored at 4°C. The bacterial cells were extracted and plated on nutrient-agar. Colonies appeared after 24 h were differentiated based on morphological observation, and again cultured on mineral

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salt-agar plate containing PCP (50 mg/l) and bromothymol blue (14). The bacterial cells extracted from the effluent of Century pulp and paper mill, Lalkua, had better capability to change bromothymol blue color into yellow were inoculated in the chemostat, and continuous enrichment was performed containing mineral salt medium and PCP (17). The media were prepared in a mineral base of following composition (g/l): Na_2HPO_4,$2H_2O$, 7.8; KH_2PO_4, 6.8; $MgSO_4$, 0.2; ammonium ferric citrate, 0.01; $Ca(NO_3)_2.4H_20$, 0.05; $NaNO_3$, 0.085; pentachlorophenol, 0.1 and trace elements solution, 1 ml/l (17). Samples of the culture were removed under aseptic conditions. The growth of the bacterial community was determined by measuring optical density at 540 nm. Samples were diluted and plated (0.1 ml/plate) on nutrient-agar.

The bacterial colonies appeared on nutrient-agar plates were morphologically characterized and purified by repeated culturing. The bacterial strains were again enriched in Erlenmeyer flasks containing mineral salt medium supplemented with pentachlorophenol (0.1 g/l) as sole source of carbon and energy, and incubated at 29°C on an orbital shaker at 150 rpm. Aliquots were transferred weekly from the culture to fresh medium. Subcultures were streaked on mineral salt medium-agar plates containing above carbon source and bromothymol blue. The strains showing higher utilization of PCP on agar plates were selected for further experiments. The strain was identified on the basis of morphological, physiological, and chemotaxonomical properties (12). Chloride molecule in culture medium was measured by the method of Bergman and Sanik (2).

The biodegradation of pentachlorophenol was determined by extraction of metabolites from the culture medium, and measuring the absorbance by spectrophotometer at 320 nm, and HPLC (12, 13). Metabolites were detected in the culture fluid after removing the bacterial cells by centrifugation at 7000 rpm for 10 min. The PCP was extracted with alkali to a pH of 12, and then with dichloromethane. The aqueous phase was removed, and acidified with HCl to a pH of <2, and extracted with dichloromethane. The process was repeated three times, and organic phase was saved each time, which was used for determination of PCP. For quantitative analysis, samples were separated by reverse-phase HPLC with a STR ODS II column (size 150 $\times$ 3.9 mm). The mobile phase was methanol and ammonium acetate buffer (0.01 M, pH 4.8) in the ratio of 70:30, v/v, flow rate was 1.5 ml/min and detected at 224 nm as described earlier (14). Percent utilization was estimated by measuring the peak area of the metabolites (17).

Mutants incapable of utilizing PCP as sole carbon source were obtained by treatment with ethidium bromide (2.5 μg/ml) for 10 cycles. In this process, cells were inoculated in nutrient broth containing ethidium bromide. Bacterial cells were removed and cultured on nutrient-agar plates. Colonies were patched on mineral salt agar plates containing PCP (0.1 g/l). The derivative could not utilize PCP was again inoculated in nutrient broth containing ethidium bromide, and finally mutants were selected based on PCP utilization. Competent cells were prepared by $CaCl_2$ method according to Sambrook *et al.* (14). In this method a single colony of *E. coli* JM109 was inoculated into Luria Broth. The cells were treated with cold $CaCl_2$ (0.1 M). Transformation of *E. coli* JM109 was performed with plasmid DNA of bacterial strain (4 μl) initially incubated in ice for 30 min, followed by heat shock for 90 s as described by Sambrook *et al.* (14).

Plasmid was isolated by modifying the method of Furukawas and Chakrabarty (4). The bacterial strain was grown overnight in mineral salt medium containing PCP (0.1 g/l). Bacterial cells were again grown in nutrient broth for overnight, and extracted by centrifugation at 7000 rpm for 10 min. Pellet was washed with TE-buffer (Tris, 0.05 M; EDTA, 0.02 M, pH 8) and incubated with lysozyme (2 mg/ml) for 3 h at 37°C with intermittent shaking. The cells were further incubated with lysis buffer containing sodium hydroxide (0.2 N) and sodium dodecyl sulfate (1%) in TE-buffer. The lysate was subjected to NaCl (3% w/v) and incubated with distilled phenol (pH 8.0). The mixture was centrifuged at 5000 rpm for 10 min and aqueous phase was treated with cold ethanol overnight at -20°C. Precipitate was recovered by centrifugation. The presence of plasmid was confirmed by restriction fragmentation as described by the manufacturer (Genei, Bangalore). The agarose gel electrophoresis was performed in agarose (0.7%) horizontal slab gel with TAE (Tris, 40 mM; sodium acetate 20 mM; EDTA 2 mM; pH 8.0) at 60 V for 2 h. The gels were stained with electrophoresis buffer containing ethidium bromide (1 μg/ml) for 20 min and visualised by UV transilluminator.

RESULTS AND DISCUSSION

Several PCP-degrading bacterial strains were isolated by enrichment culture from PCP-contaminated soils for degradation of chlorinated phenols present in the industrial effluent, but bioremediation of these compounds and identification of plasmids conferring the property of mineralization, have not been studied (13, 18). Liquid effluent from 18 industrial sites of pulp and paper mill and tannery effluent yielded 40 bacterial strains on nutrient-agar plates. In which two bacterial strains of pulp and paper mill were found to utilize a significant amount of pentachlorophenol tested on mineral salt agar plate containing PCP and bromothymol blue indicator. Sediment core containing above bacterial strains were enriched by continuous process in the chemostat. The viability of the cells was influenced by the substrate limitation in continuous culture. Initially, there was an increase in the number of bacterial cells in the chemostat, but after seven days a decline in the population of suspended cells was observed in the presence of PCP. The growth of the bacterial cells in the chemostat was determined by measuring absorbance at 540 nm and cfu on nutrient agar plates. There were changes in growth up to day 150, after that culture's turbidity remained constant till the end of the run. Results of the study indicated significant increase in number of cells and utilization of carbon source PCP (86%) as determined by spectrophotometer. A sample of culture medium taken during stabilization of growth and utilization of carbon source, on day 240, was used for structural and functional characterization of the consortium. Three different isolates were obtained on nutrient agar plates. They were characterized morphologically and biochemically, and identified as *Pseudomonas* sp. (two isolates, IST101 and IST103) and *Arthrobacter* sp. (one isolate, IST102). The strains were applied for the utilization of PCP (0.5, 1, 5, and 10 mM) in shake flask culture containing mineral salt medium. The degrading potentiality was evaluated by growth of the strains by measuring optical density at 540 nm, colonies forming unit, release of chloride, and utilization of pentachlorophenol (Fig. 1). The data indicated doubling time of, IST101, IST102, and IST103 was 1.3, 3, and 3.5 h, respectively. During utilization of carbon source stoichiometric amount of chloride was accumulated in the culture broth. Utilization of PCP determined by Spectrophotometer and HPLC. It was observed that IST103 utilized most of the PCP (84%) by 12 h, but IST102 and IST101 are able to

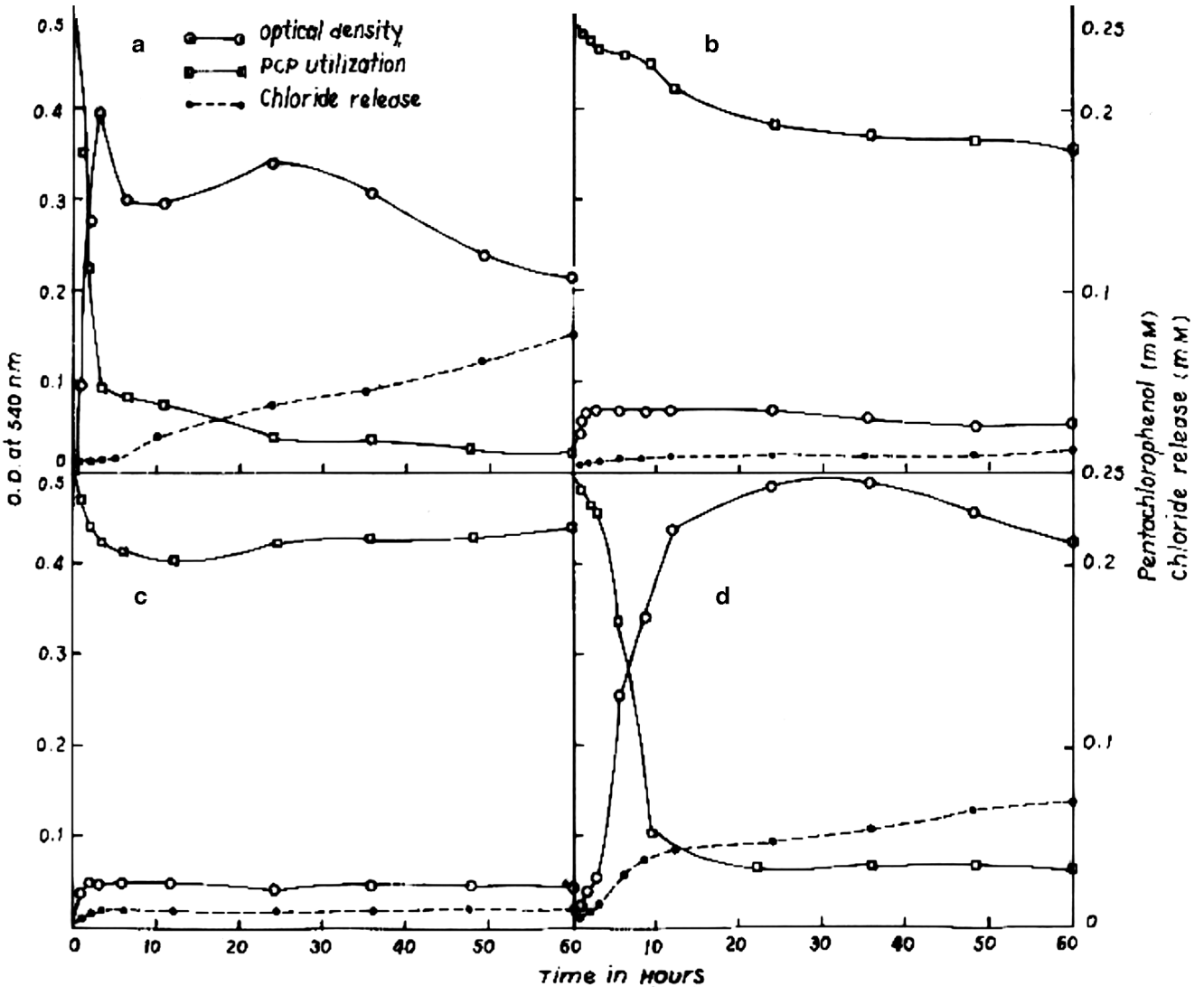

FIG. 1. Growth curve of bacterial strains, *Pseudomonas* sp. strain 103 (a), *Pseudomonas* sp. strain 103 mutant (b), *E. coli* JM109 (c), and *E. coli* JM109 transformed by low molecular size plasmid (d). Optical density is indicated as ○, PCP utilization □, and chloride release as ●.

utilize <50% PCP after 48 h. The HPLC profile of the study indicated formation of tetrachlorohydroquinone after 6 h and monochlorohydroquinone at 12 h, which reached to the maximum at 48 h by IST103 (Fig. 2).

The nature and possible role of genomic DNA in degradation of PCP has been assayed (7, 13). As can be seen in Fig. 3, two different size plasmids were observed on gel surface. The restriction digest with *Eco*RI and *Hin*dIII indicated at least ten restriction fragments in IST103. The molecular size calculated by restriction fragments size was found to be 85 kb (Fig. 3a). The data indicated that mutants produced by curing of plasmid by ethidium bromide lacked the degradation of PCP, but once low molecular size plasmid of *P.* sp. Strain103 was transferred, the derivatives could be able to utilize PCP, indicated role of plasmids in the degradation of PCP (Fig. 3b). The possible role of plasmid in degradation of PCP was further substantiated by transformation of low molecular size plasmid of strain103 into *E. coli* JM109. As can be seen in Fig. 1, the recipient of low molecular size plasmid of *E. coli* was able to degrade PCP determined by utilization of PCP, release of chloride and increase in number of bacterial cells in the culture. Data of the study indicated presence of catabolic genes in the low molecular size plasmid of the strain103. The most significant result obtained in the study was appearance of another plasmid of 2.3 kb in transformed *E. coli* JM109. Although we do not have proper explanation for the additional plasmid, it is possible that plasmid may have evolved in the transformed strain due to adaptability in PCP. In addition involvement of low molecular size plasmid in degradation of PCP has also been studied in our laboratory by shotgun cloning, and characteriza-

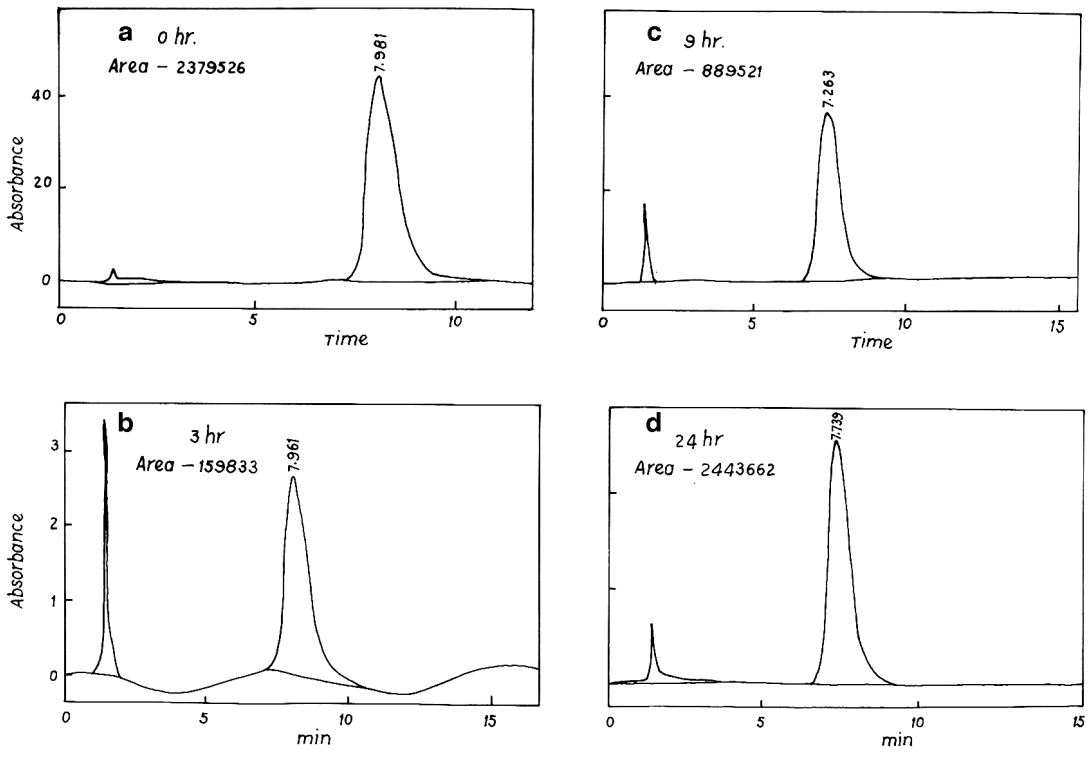

FIG. 2. HPLC profile of the metabolites extracted from the culture medium of PCP degraded by *Pseudomonas* sp. strain IST103. Conversion of PCP into tetrachlorohydroquinone (9 h) and related metabolite, chlorohydroquinone (24 h) is indicated.

tion of genes by Southern blot and sequence analysis indicated similarity with "thdf" gene of monooxygenase for degradation of thiophene and furan (data not submitted).

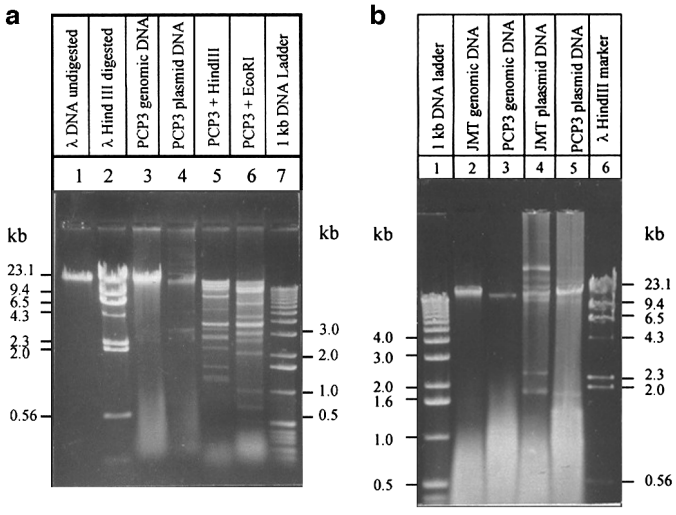

FIG. 3. Agarose gel electrophoresis of DNA of (a), *Pseudomonas* sp. strain IST 103, plasmid DNA (4), genomic DNA (3), plasmid digested from *Eco*RI (6) and *Hind*III (5), molecular size markers (1, 2, and 7), and (b) plasmid DNA of *Pseudomonas* sp. strain 103 (5), *Pseudomonas* sp. strain103 genomic DNA (3), *E. coli* JM109 genomic DNA (2), and *E. coli* JM109 transformed by low molecular size of *P.* sp. strain IST103 plasmid (4), molecular markers (1 and 6).

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