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Molecular Breeding of Oil Degrading Microorganisms: Development of Phenol Degrading Capabilities in *Pseudomonas cepacia* AC1100

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ABSTRACT

Among the large number of toxic constituents of heavier fractions of crude oil, phenols, cresols, xylenols and naphthols are prominent aromatic members. The genome of *Pseudomonas cepacia* AC1100 is a reservoir of several translocating insertion elements that have gene regulatory functions. It is also encoding genes for catabolism of chlorinated analogues of phenoxyacetic acid and chlorophenols *via* a unique pathway that has chlorohydroxy-hydroquinone as the key intermediate. On long-term enrichment with phenoxyacetic acid, we could develop a mutant of *P.cepacia* which had acquired the ability to utilize phenol. Evidence is provided indicating that phenol induces the enzymes for its degradation *via meta*-pathway that converges on the pathway of benzoate metabolism. These enzymes were not functional in the wild type AC1100. Results of complementation studies involving introduction of cloned degradative genes for chloroaromatics into mutants of *P.cepacia* which were defective in acquiring phenol degrading ability, suggest that there may be possibility of closer links of *meta*-pathway genes with the genetic material encoding degradation of chlorinated analogues.

INTRODUCTION

There has been considerable interest in recent years in bacteria which are able to degrade and detoxify the increasing amounts of aromatic compounds entering the environment as a result of man's industrial and agricultural activities. Phenol is one such aromatic compound, which acts as a major pollutant appearing in the waste products of oil refineries, coal and petrochemical industries. These compounds may also enter the environment as intermediary products *via* degradation of many pesticidal structures. Also, among the large number of toxic constituents

of heavier fractions of crude oil, phenols, cresols, xylenols and naphthols are prominent aromatic members.

The catabolic pathway for degradation of phenol in bacteria has already been elucidated in terms of their biochemistry [3]. Almost all these bacteria employ a wide range of enzymes for their initial attack on different substrates. The catabolic pathway tends to converge on just a few key intermediates, such as catechol and its substituted derivatives. This key intermediate can be further metabolized by two distinct sets of enzymes, namely those of *ortho*- cleavage pathway (β - ketoadipate pathway) and those of *meta*- cleavage pathway (α-ketoacid pathway) [3]. Phenol is usually metabolized *via* the *meta*-cleavage pathway and induction of catechol 2,3- dioxygenase can be used as an indication of the phenol being metabolized [3].

Members of *Pseudomonas* group are able to mineralize a vast majority of natural and synthetic organic compounds. This is possible because of the ability of micro-organisms to evolve new pathways or modify the existing ones to degrade new substrates by relaxing their substrate specificity [14]. We are presently studying a mutant designated as PAA, which was developed from *Pseudomonas cepacia* AC1100. The mutant PAA is able to metabolize phenol and use it as the sole source of carbon and energy. *Pseudomonas cepacia* strain AC1100 itself was initially developed under strong selective pressure in a chemostat and is the only known micro-organism with an ability to completely mineralize 2,4,5-trichlorophenoxyacetic acid [8,9]. Further, it was reported that genome of AC1100 contains insertion sequences, such as IS931 and IS932, which not only undergo transposition but also serve to activate the expression of adjacent genes [7].

In this communication, we demonstrate the activation of phenol degradative genes in *Pseudomonas cepacia* PAA and implicate the role of insertion elements. We also report the proof of degradation of phenol *via meta*-cleavage pathway, and provide evidence indicating the possibility of closer links of phenol degradative pathway genes with genetic material encoding degradation of its chlorinated analogues.

MATERIALS AND METHODS

Bacterial strains, plasmids and growth media — The strains and plasmids used in this study are listed in Table 1. Wild type (wt) AC1100 was grown and maintained on basal salt medium (BSM) containing 2,4,5-trichlorophenoxyacetic acid (1 mg/ml, BDH) and 1.5% agar for plates. *P.cepacia* PT88 (deletion mutant of AC1100) was grown and maintained on BSM containing 0.2% glucose, kanamycin (50 μ/ml) and 1.5% agar for plates. All *Pseudomonas* strains were cultured at ambient temperatures. *Escherichia coli* strains were grown on Luria Bertani broth and cultured at 37° C. Antibiotics used for *E. coli* strains included tetracycline (15 μg/ml) and kanamycin (50 μg/ml).

Table 1 — Bacterial Strains and Plasmids

Strains or plasmids	Relevant properties	Source Ref.
Strains		
E. coli AC80	*thr, leu, met, hsdR hsdM*	[1]
Pseudomonas cepacia		
1) AC1100	2, 4, 5-T$^+$ wt	[9]
2) PAA	2, 4, 5-T$^+$ *phe*$^+$ (spontaneous mutant of AC1100)	Present study
3) PT88	AC1100 :: Tn 5; Δ *chq* 2,4,5-T$^-$ Km (Tn-5 - induced deletion mutant of AC1100)	[17]
Plasmids		
1) pRK2013	Co1E1, *mob*$^+$, *tra*$^+$ Km	[5]
2) pUS1	pCP 13 Ω (*chq BamH1* 25 Kb); Tc3^r *phe*	[15]
3) pSG1	pUS1 *phe*$^+$	Present study

chq - Chlorohydroxyhydroquinone, *phe* - Phenol

Preparation of crude enzyme extract — Exponentially growing cells of *Pseudomonas* strains were harvested in a refrigerated centrifuge at 8000 rpm for 10 min. The pellet was washed in cold solution of 0.85% KCl and recentrifuged. The cells were then resuspended in chilled phosphate buffer (0.05 M, *p*H 7) containing 10% acetone (v/v). The cell suspension was then sonicated at 4°C in a Vibracell sonicator (Sonics and Material, Inc. Connecticut, USA) using a microtip probe. To prevent the enzyme from denaturation, cells were sonicated at 20 sec intervals for 4 min. The crude extract was then centrifuged at 12,000 rpm at 4°C for 30 min. A small portion of the supernatant was kept aside for protein determination. This extract was used for enzyme assay within 48 h of extraction. The extract was kept at -20°C for storage. Protein concentration was determined by Biuret method.

Enzyme assays — The catechol 2,3-dioxygenase activity was assayed as described by Nozaki [13] by measuring the increase in optical density at 375 nm due to conversion of catechol to α-hydroxymuconic-semialdehyde. The assay system in a final volume of 3 ml contained: 2.7 ml of 0.05 *M* phosphate buffer containing 10% acetone (*p*H 7); 100 µl of 10 m*M* catechol in a cuvette with 1 cm light path. The reaction was initiated by the addition of enzyme at room temperature. The rate of increase in absorbance at 375 nm was followed with a recording spectrophotometer. One unit of enzyme is defined as the amount which oxidizes 1 µmole of catechol per minute at 24°C, which corresponds to an optical density increase of 14.7 per minute at 375 nm [13].

The catechol 1,2-dioxygenase activity was assayed as described by Nakazawa [12] and modified according to Hageman [6]. The assay is based on measurement of the rate of formation of *cis, cis-* muconic acid at 260 nm. The assay mixture consisted of 4 µM EDTA; 100 µl of 10 m*M* catechol; 2.7 ml of 0.05 *M* phosphate buffer (*p*H 7) and the reaction was initiated by adding 100 µl of enzyme extract. One unit of enzyme activity is defined as that amount which catalyzes the formation of 1 µmole of *cis, cis*-muconic acid per minute at 24°C. The conversion of 1 µmole of catechol to *cis, cis*-muconic acid causes an increase in absorbance of 5.6 units at 260 nm [6].

Conjugative plasmid transfer — Bacterial triparental matings were performed as described previously [4]. The plasmid pUS1 from *E. coli* AC80 was transferred to *P. cepacia* PT88 with the help of mobilizer plasmid pRK2013 harboured in *E.coli* AC80. Transcojugants were selected by plating cells suspended in 0.85% saline on BSM plates containing tetracycline (50 µg/ml) and kanamycin (50 µg/ml) or tetracycline (50 µg/ml) and phenol (1 m*M*).

Plasmid DNA isolation — Plasmid DNA from PT88 transconjugants was isolated, as described previously [15]. Later, the plasmid was purified by cesium chloride-ethidium bromide density gradient ultracentrifugation as per Maniatis [11]. Small scale isolation of plasmids from *E. coli* was performed by alkaline lysis method [11].

Transformation — *E. coli* AC80 strains were rendered competent using a $CaCl_2$ treatment and transformation was carried out by cold shock treatment [11].

RESULTS AND DISCUSSION

Development of phenol utilizing strain

Pseudomonas cepacia AC1100 can utilize 2,4,5-trichlorophenoxyacetic acid (2,4,5-T) as the sole source of carbon and energy [9]. The 2,4,5-T degradative pathway has not been fully characterized, except for the two intermediates, *viz.* 2,4,5-trichlorophenol and 2,5-dichlorohydroxy- hydroquinone, which have been identified [2,10]. The parent strain AC1100 has the ability to metabolize 2,4,5-trichlorophenol; however, it lacks the ability to utilize its non-chlorinated analogue [10]. It is well known that in bacteria, novel catabolic pathways evolve or emerge on long-term adaptation with substrates related to those utilized by the strain. This generally occurs by broadening the substrate specificity of enzymes or by deregulation of existing genes [14]. Therefore, the possibility of strain AC1100 acquiring phenol utilizing ability on long-term adaptation was attempted.

Thick suspension of fully grown cultures of wt AC1100 and its mutant PT88 that had lost the 2,4,5-trichlorophenol utilizing ability were spread over BSM plates containing phenol or phenoxyacetic acid (1 m*M*) as the sole carbon source. None of the cultures showed growth, as they were unable to utilize non-chlorinated analogues. However, on prolonged incubation of plates for 2-3 weeks,

only the plates carrying strain AC1100 gave rise to a few colonies which grew confluently on phenol. By contrast, plates carrying the mutant PT88 did not indicate the emergence of such colonies. All the spontaneously emerged colonies from AC1100 when subcultured on fresh BSM phenol plates grew luxuriantly on phenol or phenoxyacetic acid, in addition to 2,4,5-T. This mutant was designated as *Pseudomonas cepacia* PAA. Since the parent AC1100 strain has the ability to convert 2,4,5-T acid to 2,4,5-trichlorophenol by mono-oxygenase enzyme [10], the conversion of phenoxyacetic acid to phenol in mutant PAA is presumed to be catalysed by the same enzyme. However, it is interesting that PAA has spontaneously acquired the ability to utilize phenol, which is absent in wt AC1100.

Results in Table 2 indicate the substrates utilized as sole source of carbon and energy by parental strain AC1100 and its mutants. It may be noted that AC1100 as well as its mutants utilize benzoate, irrespective of lesion in mutant PT88 in the functional pathway for 2,4,5-T degradation.

The genome of AC1100 is shown to be carrying a set of insertion sequences, such as IS931 and IS932 [7]. Both these insertion sequences are in constant translocation and transposition at different sites on host genome as well as on any incoming plasmid DNA [7]. Earlier reports have indicated that such translocation often results in activation and expression of the adjacent gene [7]. The phenol utilizing ability of mutant PAA can be attributed to an event of translocation or insertion element so as to activate any of the decisive genes that allow phenol degradation. Such activation is, however, possible only if functional genes are present on the genome of strain AC1100. This fact was proved by failure of mutant PT88 to generate spontaneous phenol degrading mutants. Mutant PT88 has a large deletion of chromosomal DNA that has resulted in loss of 2,4,5-T pathway genes [16,17].

Pathway for phenol degradation in mutant PAA

Phenol utilization in bacteria generally occurs by induction of enzymes metabolizing catechol *via meta*-cleavage pathway. Colonies of mutant PAA grown on phenol or glucose when sprayed with catechol (10 mM) exhibited distinct yellow

Table 2 — Substrates Utilized as Sole Source of Carbon and Energy by AC1100 and Its Mutants

Strains	Benzoate	2, 4, 5-T	Phenol
AC1100	+	+	–
PT88	+	–	–
PAA	+	+	+

+ growth; — no growth

colour due to breakdown of catechol to hydroxy muconic semialdehyde by catechol 2,3-dioxygenase. Both wt AC1100 and its mutant PT88 grown in benzoate or glucose did not form the yellow coloured compound. This suggested the activation of catechol 2,3-dioxygenase enzyme in mutant PAA. The specific activity of catechol 2,3-dioxygenase in cell-free extract of mutant PAA grown in glucose was compared with that of PAA grown in glucose along with various inducers, such as phenol, benzoate and salicylate.

Table 3 indicates that catechol 2,3-dioxygenase enzyme is indeed being constitutively expressed. Since the parent strain AC1100 is capable of utilizing benzoate, an active pathway to metabolize catechol, *via* catechol 1,2-dioxygenase, is expected to exist in all mutant strains described above, namely PT88 and PAA. Under such circumstances, it is natural that catechol formed from phenol in mutant PAA would be degraded by the same route. Results in Table 4 indicate that catechol 1,2-dioxygenase is indeed expressed in mutant PAA when grown on phenol, but the level of expression is less than that of PAA when grown in benzoate. Also, when the activity of catechol 2,3-dioxygenase in PAA was compared with that of the parental strain AC1100, it was found to be absent in the latter, indicating that during the adaptation of AC1100 strain on phenol, the mutant PAA arose by sudden activation of genes which otherwise were silent in the parental strain AC1100.

Table 3 — Specific Activity of Catechol 2,3- dioxygenase in PAA grown on Glucose in Presence of Different Inducers

Inducer	Specific activity (mU/mg)
None	431
Phenol	332
Benzoate	336
Salicylate	314

Table 4 — Comparison of Specific Activities of Catechol 2,3-dioxygenase and Catechol 1,2-dioxygenase in AC1100 and PAA

Organism	Substrate for growth	Specific activity (mU/mg)	
		Catechol 2, 3 dioxygenase	Catechol 1, 2-dioxygenase
AC1100	Benzoate	Nil	3566
PAA	Phenol	40	264
PAA	Benzoate	46	535

Molecular breeding of phenol utilizing strain

The degradative genes in bacteria are generally clustered and localized in distinct regions on genome or on plasmid [3]. Earlier studies have indicated that a 25 kb DNA fragment of genome of the parental strain AC1100 is encoding the functional genes necessary to metabolize 2,4,5-trichlorophenol [15]. If this region harbours any determinants for phenol degradation, any rearrangement in this region may affect the metabolism of phenol. The plasmid pUS1 carrying 25 kb fragment was transferred into mutant PT88 by triparental mating with *E.coli* AC80 [pUS1] as donor. The transconjugants were allowed to develop slowly on BSM medium with phenol as the sole source of carbon. Although the plasmid pUS1 could be transferred into PT88 at a conjugation frequency of 5×10^{-5}, the emergence of phenol utilizing transconjugants was observed at a very low frequency of 4×10^{-8}. All the phenol utilizing transconjugants were tetracycline resistant, indicating the presence of plasmid. The cells of recipient, mutant PT88 alone when used as control did not give rise to any confluently growing phenol utilizing strain. The emergence of phenol utilizing transconjugant is possible only if the 25 kb fragment has undergone a genetic rearrangement or a mutation that allows degradation of phenol.

To confirm that the rearranged 25 kb fragment from pUS1 was linked to phenol utilization, the plasmid from its transconjugants was isolated and purified by cesium chloride- ethidium bromide density gradient centrifugation. This plasmid, isolated from PT88 transconjugant, was designated as pSG1. The plasmid preparation was used to transform the competent cells of *E. coli* AC80 and the resultant *E. coli* AC80 [pSG1] was used as donor strain to transfer pSG1 back into mutant PT88 by triparental mating. The results indicated that pSG1 could indeed generate phenol utilizing transconjugants at a frequency of 1×10^{-5} per donor cell, comparable to plasmid transfer frequency, indicating that a rearrangement has occurred in the fragment. A similar rearrangement might have helped the mutant PAA to acquire phenol utilizing ability.

CONCLUSION

Members of *P. cepacia* group utilize maximum number of carbon compounds among those known to be metabolized by other bacteria. The versatility of such strains is largely decided by a set of repeated sequences on the host genome, which acts as insertion sequences capable of activation or translocation of catabolic genes. Two interesting aspects have been dealt with in this paper. The trichlorophenoxyacetic acid utilizing strains could be trained to degrade non-chlorinated phenol, after long- term adaptation. It is evident that the event of rearrangement or mutation has directed the synthesis of a key enzyme catechol 2,3-dioxygenase, which is required to degrade phenol. Secondly, we could show that a similar breeding could be achieved at molecular level by maintaining the 25 kb DNA fragment implicated in trichlorophenol utilization into host PT88.

Whether the genes directing phenol utilization are a result of translocation or activation by insertion element from host genome is not clear at this stage. Precise genetic mapping of pSG1 should give a better understanding.

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