CHAPTER V

MOLECULAR REARRANGEMENT RESPONSIBLE FOR EMERGENCE OF STRAIN PAA FROM P. CEPACIA AC1100
Chapter IV revealed the potential of mutant strain PAA in biodegradation of phenol by continuous cultures. The ability of mutant strain PAA to degrade phenol in LSM indicates the possibility of it being used in degradation of phenol from aquatic/estuarine water system.

The present chapter deals with molecular characterization of an event which resulted in the development of phenol-degrading mutant PAA from strain AC1100 as observed in Chapter II. Strain AC1100 was isolated by long term chemostat selection using Plasmid Assisted Molecular Breeding (Kellogg et al., 1981). Repeated sequences in AC1100 have been known to activate genes by reallocation of genetic sequences and are suspected to have played a role in sequestering diverse aromatic degrading genes, like those involved in 2,4,5-T utilization, from other bacterial genomes (Tomasek et al., 1989; Haugland et al., 1990). The studies conducted in present chapter will help us to understand the molecular mechanism involved in the activation of phenol-degrading ability, which was otherwise silent in parent strain AC1100.
**Materials & Methods**

**Chemicals**

Agarose was purchased from Himedia Ltd. Restriction enzymes were obtained from Genei Chemical Co. Ltd., Bangalore. Cesium chloride was of analytical grade and was purchased from SRL SISCO. 2,4,5-T was obtained from Sigma. p-toluidine was obtained from Pune Chemical Co. Ltd., whereas Phenol crystals (molecular biology grade) were purchased from SRL SISCO Ltd. Dialysis tubing was obtained from Sigma. All other chemicals used for preparation of buffers and media were either of LR or AR grade and purchased from local suppliers.

**Micro-organisms:**

Strain PAA used in the experiments was obtained from *P. cepacia* AC1100 as mentioned in Chapter II. Strain AC1100, PT88 and *E. coli* AC80 were obtained from Dr. Anand Chakrabarty, University of Illinois, USA. PT88[pUS1], PT88[pUS105] and PT88[pSG1] were obtained
during the course of present study. The genotype of these strains are mentioned in Table 5.1.

Plasmids

Plasmid pUS1 and pUS105 were obtained from Dr. Anand Chakrabarty, USA. Plasmid pSG1 was obtained during the course of present studies. Characteristics of these plasmids are mentioned in Table 5.1.

Growth of micro-organisms:

_P.cepacia_ AC1100 and its mutant strains PT88 and PAA were grown and maintained as mentioned in chapter II. _E.coli_ AC80 was routinely grown in LB medium on rotary shaker at 150 rpm at 30±2°C. _E.coli_ AC80 transformants i.e., AC80 [pUS1]; AC80 [pUS105] and AC80 [pSG1] were grown in LB medium under selective pressure using tetracycline at a concentration of 15 μg/ml. AC80 transformants were routinely maintained in LB stabs at 4°C. For inoculation purpose, AC80 transformants were cultured on LB agar plates containing 15 μg/ml
<table>
<thead>
<tr>
<th>Strains/Plasmids</th>
<th>Relevant Properties</th>
<th>Source/Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Strains</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1) PT88</td>
<td>AC1100::Tn5; chq^ -</td>
<td>Tomasek et al., 1986.</td>
</tr>
<tr>
<td></td>
<td>2,4,5-T, Kan^ r</td>
<td></td>
</tr>
<tr>
<td>2) PT88[pUS1]</td>
<td>AC1100::Tn5; chq^ +</td>
<td>Present study</td>
</tr>
<tr>
<td></td>
<td>2,4,5-T; kan; Phe; tet^ r</td>
<td></td>
</tr>
<tr>
<td>3) PT88[pSG1]</td>
<td>AC1100::Tn5; chq^ +</td>
<td>Present study</td>
</tr>
<tr>
<td></td>
<td>2,4,5-T; kan; Phe; tet^ r</td>
<td></td>
</tr>
<tr>
<td>4) PT88[pUS105]</td>
<td>AC1100::Tn5; chq^ +</td>
<td>Present study</td>
</tr>
<tr>
<td></td>
<td>2,4,5-T; kan; Phe; tet^ r</td>
<td></td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1) pRK2013</td>
<td>ColE1, mob, tra, Km</td>
<td>Dr. Anand Chakrabarty, University of Illinois, USA</td>
</tr>
<tr>
<td>2) pUS1</td>
<td>pCP 13^N(chq BamHI 25kb); Tet^-</td>
<td>Sangodkar et al., 1988</td>
</tr>
<tr>
<td></td>
<td>r phe^-</td>
<td></td>
</tr>
<tr>
<td>3) pUS105</td>
<td>pCP 13^N(chq BamHI 8kb); Tet^-</td>
<td>Sangodkar et al., 1988</td>
</tr>
<tr>
<td></td>
<td>r phe^-</td>
<td></td>
</tr>
<tr>
<td>4) pSG1</td>
<td>pUS1 phe^ +</td>
<td>Present study</td>
</tr>
</tbody>
</table>

*chq* - Chlorohydroxyhydroquinone, *phe* - phenol
tetracycline and later inoculated in LB medium containing tetracycline (15 μg/ml). *P. cepacia* PT88 was grown in BSM containing 0.2% glucose and 50 μg/ml kanamycin.

**Triparental mating:**

The donor plasmids pUS1 and pUS105 from *E. coli* AC80 were transferred to recipient *P. cepacia* PT88 with the help of mobilizer plasmid pRK2013 residing in *E. coli* AC80. The ratio of donor:helper:recipient was 10:1:1. *E. coli* AC80 strains containing pUS1 and pUS105 were grown in LB medium containing 15 μg/ml tetracycline for 15 h on rotary shaker at 150 rpm at 30°C. Strain PT88 was grown for 36 h in BSM supplemented with 0.2% glucose and kanamycin (50 μg/ml) on a rotary shaker at 150 rpm at 30±2°C. Cell suspension of donor, recipient and helper was filtered aseptically through a 0.22 μm filter. The filter paper was washed twice with 2 ml of sterile LB. The filter paper was incubated on LB agar plate at 30°C for 24 h and was aseptically transferred to a sterile centrifuge tube. The cells on the filter paper were flushed with 0.85% KCl. The
cell suspension was centrifuged at 10,000 rpm for 10 minutes at 4°C. The cell pellet was washed twice with 0.85% KCl and finally resuspended in 1 ml of 0.85% KCl.

PT88 transconjugants were selected by plating the above mixture on BSM agar plates containing 75 µg/ml tetracycline, 50 µg/ml kanamycin, supplemented with 1mM phenol or 0.2% glucose as sole carbon source. Washed cells of strain PT88 were used as control by plating a concentrated cell suspension on BSM agar plates containing tetracycline, kanamycin and phenol. Viable cell count of recipient PT88 was obtained by serial dilutions and plating on BSM agar plates containing 0.2% glucose and kanamycin (50 µg/ml). Plasmid transfer frequency was calculated as the ratio of number of transconjugants obtained on BSM agar plates containing tetracycline (75 µg/ml), kanamycin (50 µg/ml) and glucose (0.2%) to viable cell count of recipient (PT88) cells.

Phe transconjugants obtained during triparental mating were checked for ability to grow on BSM agar plate supplemented with 0.2% glucose and kanamycin (50 µg/ml). Further, PT88 transconjugants growing on BSM agar plate with 1 mM phenol as carbon source under
selective pressure of tetracycline (75 μg/ml) and kanamycin (50 μg/ml) were then confirmed by streaking on BSM agar plates supplemented with 0.1% glucose and 0.08% 2,4,5-T.

**Plasmid DNA isolation:**

Plasmid DNA from PT88 transconjugants were isolated as described previously (Sangodkar et al. 1988). The plasmids were further purified by Cesium chloride-ethidium bromide density gradient ultracentrifugation as mentioned by Sambrook et al. (1989). Small scale plasmid isolation from *E. coli* strain AC80 was done by alkaline lysis method (Sambrook et al. 1989).

**Transformation:**

*E. coli* AC80 strains were rendered competent using a CaCl$_2$ treatment and transformation was carried out by heat shock treatment (Sambrook et al. 1989).
Detection of catechol by p-toluidine in PT88 transconjugants:

Chromogenic detection of catechol was done using p-toluidine as mentioned in Chapter II. BSM agar plates were prepared supplemented with 1 mM phenol. FeCl₃ was added aseptically to BSM after sterilization at a final concentration of 1.5 mM. PT88, PT88 pUS1 and PT88 pSG1 were patched on the 3 corners of BSM agar plate which was previously plated with 40 μl of 50 μg/ml p-toluidine. 10 μl of 0.01 mM catechol was also patched as positive control. The plate was sealed with parafilm and incubated at 30°C for 2 weeks.

Growth of PT88 [pSG1] on phenol:

Colonies of PT88 [pSG1] transconjugants from BSM tetracycline, kanamycin phenol agar plates were inoculated in 100 ml of BSM solution containing 50 μg/ml kanamycin and 1 mM phenol as sole carbon source and incubated at 30°C on rotary shaker (150 rpm). The cell growth was monitored at 560 nm and phenol concentration in the medium was determined by modified aminoantipyrine
method (Chapter III). 1 ml of the culture was taken from the flask aseptically at defined time intervals for the above measurements.

Oxygen uptake studies with resting cell suspension of PT88 [pSG1] and PT88 [pUS1] transconjugants:

Oxygen uptake by resting cell suspension of PT88, PT88 [pUS1] and PT88 [pSG1] were determined by Gilson 5/6 oxygraph equipped with a Clarke electrode. Endogenous oxygen uptake was determined before addition of phenol or catechol. Corrected oxygen uptake was expressed as nmole of O2/min/mg cells. Phenol was used at a concentration of 0.01% whereas catechol was used at a concentration of 0.05%. Resting cells were prepared as mentioned in Chapter II. PT88 [pUS1] tranconjugant and PT88 were grown in BSM containing kanamycin (50 μg/ml), tetracycline (75 μg/ml), glucose (0.2%) and BSM containing kanamycin (50 μg/ml), glucose (0.2%) respectively. PT88 [pSG1] was grown in BSM containing 0.1% glucose, 1 mM phenol and under selective pressure of 50 μg/ml kanamycin and 75 μg/ml tetracycline.
Restriction analysis of plasmid pUS1 and pSG1:

Plasmid DNA obtained by alkaline lysis as per Sambrook et al. (1989) was digested with restriction enzymes. BamHI and EcoRI were used for restriction digestion of plasmids as per Sambrook et al. (1989). The samples were electrophorosed on 0.7% agarose gel as per Sambrook et al. (1989). A DNA precut with Hind III was used as standard marker for determination of molecular weights of restricted DNA fragments.

Results

Manipulation of mutant strain P. cepacia PT88 to acquire phenol utilizing phenotype:

Strain PT88 is a deletion mutant of P. cepacia AC1100 that lacks the ability to degrade 2,4,5-T and 2,4,5-Trichlorophenol. As seen in Chapter II, no spontaneous mutants emerged from strain PT88 even on prolonged incubation in presence of phenol. The strategy to manipulate PT88 to utilize phenol, involved
transfer of plasmids carrying genomic regions of wt AC1100 by conjugation and complementing the deletion + mutation to phe by forcing the transconjugants to grow in presence of phenol as done for the emergence of PAA from strain AC1100. The assumption was that plasmids maintained in PT88 will possibly be prone to similar genetic rearrangement as occurred in strain PAA. Plasmid [pUS1] and [pUS105] (Fig.5.1) carrying chq genes on pCP13 cosmid replicon were used and tetracycline resistant transconjugants were selected with glucose as carbon source. The transconjugants were designated as PT88 [pUS1] and PT88 [pUS105]. Forced adaptation of PT88 [pUS1] and PT88 [pUS105] to utilize phenol were carried out using two protocols.

In the first protocol transconjugants were picked from BSM tetracycline Kanamycin, glucose plates and patched by using a heavy inoculum of transconjugants on BSM agar plates containing phenol and incubated for 4 weeks to check for phenol utilizing colonies. In the second protocol, 0.1 ml of transconjugants after mating were plated and allowed to develop slowly on BSM agar medium with 1 mM phenol as sole source of carbon. Plasmid pUS1 and pUS105 could be transferred into PT88
Fig. 5.1  Restriction map of 25 kb insert of pUS1 and 8 kb insert of pUS105. Open box indicates the location of chq gene.
at a conjugation frequency of $5 \times 10^{-5}$ and $9 \times 10^{-5}$.

However, no immediate emergence of PT88 transconjugants either with pUS1 or pUS105 were observed, when cells were patched on phenol plates. Experiments using second protocol, wherein the mated mixture were plated directly on phenol plates, few phenol utilizing (Phe) colonies from transconjugant PT88 [pUS1] were found to emerge after four weeks. Further incubation of these plates for 2-3 days, resulted in increase in the colony size. The emergence of phenol utilizing transconjugants were observed at a low frequency of $4 \times 10^{-8}$. All the phenol utilizing transconjugants were tetracycline resistant indicating the presence of plasmid. The PT88 [pUS1] transconjugants growing on phenol were stable and could be propagated on phenol plates through successive cultures (Fig. 5.2).

+ No Phe colonies emerged on BSM agar phenol plates in case of transconjugants PT88 [pUS105] even on prolonged incubation. Few very minute colonies emerged, which when restreaked on phenol plates did not promote growth. Further, the cells of recipient, mutant PT88 alone when used as control did not give rise to any confluently growing phenol utilizing strain on BSM agar
Fig. 5.2  Growth of PT88 [pSG1] transconjugants in BSM containing tetracycline (75 µg/ml); Kanamycin (50 µg/ml) and phenol (1 mM).
plates containing kanamycin, tetracycline and phenol + indicating that the Phe ability acquired by transconjugants PT88 [pUS1] was by virtue of presence of pUS1, which had presumably undergone a rearrangement.

Confirmation of mutation of pUS1 in PT88:

One of Phe, PT88 [pUS1] transconjugant clone which was rapidly growing on phenol was grown in 500 ml of BSM containing tetracycline (75 μg/ml), glucose (0.1%) and phenol (1 mM) for 48 h. The plasmids from PT88 transconjugants was isolated and purified by Cesium chloride density gradient centrifugation. The plasmid pUS1 obtained from PT88 transconjugant growing on phenol was designated as pSG1. The plasmid preparation was used to transform the competent cells of E. coli AC80. The tetracycline resistant transformants of AC80 were obtained.

The resultant E. coli AC80 [pSG1] was now used as a donor strain to transfer the plasmid back into mutant strain PT88 by triparental mating. The PT88 transconjugants were selected on BSM agar plates.
containing tetracycline, kanamycin and glucose as well as on BSM agar plates containing tetracycline, kanamycin and phenol. The plasmid transfer frequency was $1 \times 10^{-5}$ as observed on BSM glucose plates containing tetracycline and kanamycin. Similarly the transconjugants selected on BSM phenol plates were obtained at a frequency of $4 \times 10^{-5}$ which was $10^3$ fold higher than that obtained during previous transfer, confirming that the Phe phenotype acquired by PT88 transconjugant was due to mutation in pUS1. The rearranged plasmid was therefore designated as pSG1.

Utilization of aromatic substrates by transconjugants PT88 [pUS1] and PT88 [pSG1], showed a characteristic bright red colour formation when streaked on BSM agar plates containing glucose and 2,4,5-T (Fig. 5.3). Similarly, PT88 which did not grow on 2,4,5-T, could utilize it as sole source of carbon, when its transconjugants carried either plasmids pUS1, pSG1 or pUS105. Further, like PT88, all its transconjugants showed kanamycin resistance and utilized benzoate as sole source of carbon.
Fig. 5.3 Ability of PT88 [pSG1] transconjugants to form red colour intermediate, 5-chloro,2-hydroxy-1,4-benzoquinone, when grown in presence of glucose and 2,4,5-T.
Kinetics of phenol utilization by PT88 [pSG1]:

Since amongst the transconjugants only PT88 [pSG1] could grow on phenol, pure colonies of PT88 [pSG1], pregrown on BSM agar plates containing tetracycline, kanamycin and phenol were picked and inoculated in 100 ml of BSM solution containing kanamycin and 1 mM phenol. Figure 5.4 shows the typical growth pattern of PT88 [pSG1]. There was a lag of almost 16 h before active growth ensued. There was exponential increase in cell mass as observed by increase in cell absorbance at 560 nm with simultaneous depletion of phenol from the medium as seen from Figure 5.4. Phenol was completely degraded from 100 ppm to a final concentration of 3 ppm. When PT88 [pUS1] and PT88 [pUS105] were used as control, no growth was observed in BSM kanamycin phenol, even after addition of high inoculum. Strain PAA was grown separately in BSM containing phenol, as control (Fig. 5.4).
Fig. 5.4 Batch culture of PT88 [pSG1] (---) and PAA (-----) in BSM containing 1 mM phenol. Strain PAA was grown in separate flask for comparing the growth kinetics with PT88 [pSG1] transconjugants. ○ - Growth of PAA and PT88 [pSG1]; ● - phenol concentration.
Biochemistry of phenol utilization by PT88 [pSG1]:

a) Detection of catechol as an intermediate of phenol degradation:

To detect formation of catechol, a key intermediate of phenol, PT88 [pSG1] colonies were patched on BSM agar plates containing FeCl₃ and phenol as mentioned in Chapter II. p-toluidine was used for chromogenic detection. PT88 [pSG1] colonies became darkish brown in colour in presence of p-toluidine as seen in Figure 5.5. Catechol used as control also showed dark brown spots. Strain PT88 and its transconjugants when used as control did not show any colour formation (Fig. 5.5) indicating that PT88 [pSG1] generated catechol from phenol. Colonies of PT88 [pSG1] when sprayed with catechol, however, did not show formation of any yellow colour, indicating distinct absence of α-hydroxymuconic semialdehyde in the pathway. Also, crude extracts prepared from transconjugants PT88 [pSG1] pregrown in BSM phenol did not show any C230 activity.
Fig. 5.5 Amplification of catechol detection by incorporation of p-toluidine in the medium. The petriplate has been divided into 4 sectors and bacterial cells were inoculated as patch. (1) PT88; (2) PT88[pUS1]; (3) PT88[pSG1] and (4) catechol.
Detection of phenol degrading enzymes:

To detect phenol hydroxylase activity, washed cells of PT88 [pSG1] pregrown in phenol were incubated with phenol. Table 5.2 shows the results of oxygen uptake studies done using PT88 and its transconjugants in presence of phenol. It can be clearly seen that PT88 [pSG1] transconjugants indeed oxidized phenol without any lag. The oxygen uptake by PT88 [pSG1] transconjugants is nearly identical to that observed in case of PAA (Chapter II, Table 2.3). In case of PT88 and its transconjugants PT88 [pUS1], oxidation of phenol was negligible as seen from Table 5.2. Strain PT88 and all other transconjugants were seen to oxidize catechol at the same rate.

Genetic analysis of PT88 [pSG1]:

After confirmation of the ability of PT88 [pSG1] transconjugants to grow on phenol, plasmids pUS1 and pSG1 were isolated from respective E. coli AC80 transformants and analyzed by gel electrophoresis. Figure 5.6 depicts the DNA profile.
Table 5.2  Oxygen uptake nmole O$_2$/min/mg cell

<table>
<thead>
<tr>
<th>Strain</th>
<th>Phenol</th>
<th>Catechol</th>
</tr>
</thead>
<tbody>
<tr>
<td>PT88 [pSG1]</td>
<td>18.05</td>
<td>34.51</td>
</tr>
<tr>
<td>PT88</td>
<td>&lt; 1</td>
<td>28.7</td>
</tr>
<tr>
<td>PT88[pUS1]</td>
<td>0</td>
<td>10.2</td>
</tr>
</tbody>
</table>
Fig. 5.6  Plasmid profile from _E. coli_ AC80 [pUS1] and AC80 [pSG1] on a 0.7% agarose gel. Lane 1. plasmid DNA from AC80 [pUS1]; Lane 2. plasmid DNA from AC80 [pSG1].
Figure 5.7 depicts the BamHI digest of plasmid pUS1 and using the standard molecular weight markers of DNA cut with Hind III, the molecular weight of pUS1 digested fragments were 21, 13.4, 5, 3.8 and 2.8 kb, respectively. The EcoRI digest of plasmid pUS1 resulted in 21, 6.5, 5.8, 4.3, 4, 3, 1.4, 0.56 and 0.55 kb, respectively (Fig. 5.8).

Figure 5.9 shows the profile of EcoRI digest of plasmid pSG1. Fragments with molecular weights 21, 3, 2.5 and 2.2 kb could be observed. Plasmid pSG1 when treated with BamHI showed only one linearised band.

DISCUSSION

In the previous studies, we have reported the evolution of mutant PAA from *P. cepacia* AC1100 by growing it under selective pressure in presence of phenoxyacetic acid and phenol. Enzymatic studies also revealed the presence of an active enzymatic machinery in strain PAA, which is independent of the 2,4,5-T pathway and is involved in phenol metabolism (Ghadi & Sangodkar, 1994). Surprisingly, strain AC1100 or PT88
Fig. 5.7  Restriction analysis of pUS1. Lane 1. pUS1 plasmid DNA from AC80 digested with Bam HI. Lane 3. λDNA digested with Hind III. Molecular weight of fragments from top to bottom: 23, 9.4, 6.5, 4.3, 2.3 and 2 kb.
Fig. 5.8 Restriction analysis of pUS1. Lane 1. λ DNA digested with Hind III. Molecular weight of fragments from top to bottom: 23, 9.4, 6.5, 4.3, 2.3 and 2 kb. Lane 2. plasmid pUS1 DNA digested with EcoR I.
Fig. 5.9 Restriction analysis of pSG1. Lane 1. λ DNA digested with Hind III. Molecular weight of fragments from top to bottom: 23, 9.4, 6.5, 4.3, 2.3 and 2 kb. Lane 2. Plasmid pUS1 digested with BamHI. Lane 3 and 4. plasmid pSG1 DNA from AC80 digested with EcoRI.
did not show the presence or any evidence of enzymes which are involved in phenol catabolism.

Members of *Pseudomonas* species, especially *P. cepacia* is the most biochemically versatile organism. The ability to evolve new pathways, as reviewed by Coco et al, (1990) and Meer et al, (1992) has conferred *Pseudomonas* with the unique ability to mineralize vast majority of natural and synthetic organic compounds. *P. cepacia* AC1100 is a unique organism created and evolved by Plasmid Assisted Molecular Breeding in a chemostat (Kellogg et al., 1981). Strain AC1100 degrades 2,4,5-T via 2,4,5-trichlorophenol and chlorohydroxyquinone (Karns et al., 1983; Chapman et al., 1987). The forced evolution of strain AC1100 to give rise to strain PAA (Chapter II) could be possible either by modifying key enzymes of the existing 2,4,5-T pathway to accommodate non-chlorinated phenol or by activation of silent or cryptic phenol degrading genes.

The presence of phenol hydroxylase and *meta*-pathway enzymes in PAA and its absence in strain AC1100 and PT88 indeed confirms the phenomenon of gene activation which occurs during selective pressure of AC1100 as mentioned
before (Chapter II). To characterize the molecular mechanism leading to emergence of PAA, the following studies were carried out.

Strain AC1100 harbors at least two plasmids (Ghosal et al., 1985), one of which shows considerable homology to 2,4-D degradative plasmid pJP4 (Don and Pemberton, 1983). A substantial number of aromatic catabolic pathways are plasmid encoded. There are few reports on catabolic plasmids responsible for phenol degradation (Shingler et al., 1989; Hermann et al., 1987 and Kivisaar et al., 1989). However, _P. putida_ strain PP1-2 is known to degrade phenol by ortho cleavage pathway which is chromosomally encoded (Wong and Dunn, 1974). Hence strain PAA was conjugated with a Phe strain PT88. Our experiments indicate the possibility that the two plasmids present in AC1100 are not directly responsible for phenol degradation. Also continuous subculturing of strain PAA in glucose has revealed no loss in phenol degrading characteristics of strain PAA as observed by Hermann et al (1987) in case of _P. putida_. Thus the phenol degrading genes in strain PAA are chromosomally encoded.
The meta-cleavage pathway genes are generally plasmid encoded. A fraction of mixed cell suspension of strain PAA and PT88 was incubated for 24 hours for biparental mating. 0.1 ml of cell suspension was spread plated on BSM plated containing kanamycin (50 ug/ml) and 0.2% glucose, so as to obtain a dense growth. The colonies were spread with 10 mM catechol. No yellow colour colonies were observed when sprayed with catechol indicating the possibility of both phenol degrading and meta-cleavage pathway genes to be encoded chromosomally in strain PAA.

Further confirmation of phenol degrading genes being chromosomally encoded came interestingly by complementation of 25 kb fragments from AC1100 which is known to complement 2,4,5-T degradation in PT88 (Sangodkar et al, 1988). During triparental mating, though plasmid transfer frequency was $5 \times 10^{-5}$ as observed on BSM agar plates containing tetracycline, kanamycin and glucose, initially no transconjugants could be obtained on phenol. However, on prolonged incubation, few PT88 [pUS1] transconjugant colonies started emerging on phenol plates. The emergence of such phenol utilizing transconjugants from pUS1 was
observed at a very low frequency of $4 \times 10^{-8}$.

The situation observed during emergence of PT88 transconjugants growing on phenol is analogous to emergence of mutant strain PAA from AC1100, when the latter was grown under selective pressure in presence of phenol. The emergence of strain PAA from AC1100 took about 3 weeks and occurred at a frequency of $1 \times 10^{-8}$. PT88 which has a deletion mutation did not give rise to phenol degrading variants. The similarity in both the experiments point out to the possibility that the molecular mechanism resulting in gene activation in PAA and PT88 [pSG1] seems analogous.

pSG1 plasmid was isolated from PT88 background and was transformed back into *E. coli* strain AC80. The resultant *E. coli* AC80 [pSG1] was used as a donor strain to transfer pSG1 back into strain PT88 by triparental mating. The results indicate that pSG1 could indeed generate phenol utilizing transconjugants at a frequency of $1 \times 10^{-5}$, which was comparable to plasmid transfer frequency and was 10 fold higher than the frequency with which the Phe emerged from PT88 [pUS1] indicating that all the pSG1 plasmids transferred the Phe.
phenotype to PT88. Thus, unlike pUS1, pSG1 contains the rearranged or mutated fragment which is solely responsible for high number of Phe transconjugants.

The PT88 [pSG1] transconjugant was stable only when grown under selective pressure in presence of tetracycline and phenol. Strain PT88 is a deletion mutant of AC1100 (Tomasek et al., 1986) and is a blocked mutant for 2,4,5-T degradation. In presence of glucose and 2,4,5-T, it accumulated a bright red coloured intermediate (Sangodkar et al., 1988). This test was used to confirm the identity of transconjugants as PT88. All Phe transconjugants accumulated bright red compound which was less intense than strain PT88 because of complementation of chq mutation. pUS1 also complements 2,4,5-T mutation in PT88 by producing CHQ metabolizing enzymes (Sangodkar et al., 1988).

It may be recalled from previous work of Sangodkar et al. (1988) that the 4.8 kb XbaI-BamHI fragment of pUS1028 containing CHQ metabolizing genes of 2,4,5-T pathway fails to hybridize with BamHI digested DNA from PT88 indicating deletion in this region and also possibly in the lower part of the pathway. Since pUS1
containing 25 kb fragment shows Phe complementation after getting rearranged to pSG1, it clearly indicates that the fragment contains the genes responsible for initial conversion of phenol to catechol. The genes responsible for conversion of phenol to catechol is not related to 2,4,5-T degrading genes as pUS105 which exactly complements 2,4,5-T mutation in PT88 (Sangodkar et al., 1988) did not give rise to Phe transconjugants. Also PT88 failed to give rise to any Phe mutants indicating a possible deletion in genes responsible for conversion of phenol to catechol. The above observation confirm that initial part of phenol degradation is chromosomally encoded and the fragment responsible is present on plasmid pSG1.

The Phe transconjugants obtained when sprayed with catechol, did not form yellow colour. Even crude extracts prepared by sonication of PT88 [pSG1] transconjugants pregrown with phenol as sole source of carbon did not show any catechol 2,3-dioxygenase activity. This suggests that the 25 kb fragment contains only the active phenol hydroxylase gene.

PT88 [pSG1] accumulates catechol as intermediate,
when grown in presence of phenol which interacts with p-toluidine to form a brown coloured intermediate (Fig. 5.5). Detection of catechol as an intermediate using p-toluidine has been widely used by Parke (1993). PT88 [pSG1] however, did not accumulate any brown colour intermediate as such when grown in presence of phenol (in absence of p-toluidine). Mutants defective in catechol metabolism, generally turn brown. The presence of complementation and absence of accumulation of brown colour intermediate indicates the channeling of catechol via ortho pathway which is preexisting in PT88. A similar phenomena has been observed in Pseudomonas CF600 which degrades phenol via meta cleavage pathway (Shingler et al. 1989). A Tn5 mutant of CF600 having lost the ability of metabolizing phenol could be complemented by 1.2 kb fragment which encoded for phenol hydroxylase. However, the catechol which was formed was channelized via the ortho-pathway which was chromosomally located in strain CF600 (Shingler et al., 1989).

Unlike pUS1, pUS105 fails to complement Phe PT88, we conclude that, the presence of 16 kb fragment to the right of CHQ metabolizing genes, is essential for
activation of phenol degrading genes. However, the exact role could not be determined. Also, failure to obtain any positive indication of meta-cleavage activity from pUS1 or pSG1 might indicate the possibility of existence of meta-cleavage pathway genes elsewhere in the genome.

Clustering of aromatic pathway genes on chromosome or plasmid have been well documented (Bartilson et al., 1990; Shingler et al., 1989; Kivisaar et al., 1989; Kukor and Olsen, 1991). However, our results differ from above. Strain AC1100 has been isolated by Plasmid Assisted Molecular Breeding in a chemostat by breeding a mixed population of bacteria from diverse polluted sites. The creation of P. cepacia AC1100 with 2,4,5-T degrading ability can be attributed to gene flux taking place amongst the microorganism. Many aromatic catabolic pathways are either plasmid encoded or found to be either wholly or partially encoded within large transposon (Meer et al., 1992). The potential degrading ability must have been built up piece meal by acquisition of preevolved metabolic modules either from Pseudomonas or non Pseudomonas ancestor. Hence strain AC1100 might possess a reservoir of genes, homologous or non homologous. Also, the genetic location of
catabolic pathway may be different.

The 2,4,5-T genes located on pUS1 are unique to *P. cepacia* AC1100, as none of the DNA from other *Pseudomonas* species showed any hybridization indicating the acquisition of 2,4,5-T degrading genes from non-*Pseudomonas* ancestor (Tomasek et al., 1989).

The PT88 [pSG1] transconjugants indeed metabolize phenol as seen from batch culture experiments. Similarly oxygen uptake experiments indicate higher phenol oxidation rate by PT88 [pSG1] transconjugants, when compared to PT88 [pUS1] transconjugants. The values are comparable to oxidation rate of PAA pregrown in phenol (chapter II, table 2.3).

EcoRI digestion of pSG1 resulted in fragments having molecular weight 21, 3, 2.5 and 2.2 kb respectively. By contrast, many fragments detected during digestion of pUS1 by EcoRI, could not be observed in pSG1. For instance, 6.5, 5.8, 4.3, 4, 1.4 were not represented in pSG1. Besides the vector arm of pCP13, only 3 kb-EcoRI fragment was observed to be common in both, pUS1 and pSG1. The presence of 2.5 and 2.2 kb in
pSG1 were additional and were not detected during EcoRI digest of pUS1. These observations clearly provide an evidence that the pSG1 has resulted after the rearrangement of pUS1.

A significant conclusion which can be drawn to explain the variation in restriction pattern of pSG1 is the molecular rearrangement of 25kb fragment in pUS1 (Phe ) to pSG1 (Phe ). This involves deletion of DNA from pUS1, during growth of PT88 [pUS1] transconjugants in presence of phenol.

Strain AC1100 and PT88 is a reservoir of repeat sequences and both IS931 and IS932 have been found to be mostly clustered around 1300 kb or one third of chromosomal DNA. (Haugland et al. 1990; Coco et al. 1990). Also three copies of IS931 were mapped near 2,4,5-T genes encoding degradation of 5-chloro-1,2,4-trihydroxybenzene, an intermediate in the degradation of 2,4,5-T. (Tomasek et al. 1989).

IS931 is known to undergo transposition in AC1100 and can also serve to activate the expression of promoterless genes and mobilise adjacent DNA (Haugland
et al. 1989). Figure 5.10 shows the relative position of three copies of IS931 sequences adjacent to \textit{chq} genes in pUS1. Similarly pUS105, a subclone of pUS1 which complements 2,4,5-T mutation in PT88 also has a single copy of IS931 as seen in Figure 5.10. (Tomasek et al., 1989).

The 3 kb-EcoRI fragment seen in restriction pattern of pUS1 and pUS105, has also been observed in pSG1 (Fig.5.9). However, this entire 3 kb fragment is not directly involved in 2,4,5-T utilization in pUS1 and pUS105 because pUS1028, a subclone of 25 kb fragment from pUS1, complemented 2,4,5-T mutation in PT88 though there was an internal deletion of 1.1 kb inside the region of 3 kb fragment. (Sangodkar et al. 1988). Thus, the presence of 3 kb-EcoRI fragment seems to be directly responsible for phenol utilization or activation. The presence of IS931 sequence just adjacent to the 3 kb-EcoRI fragment may have a very crucial role to play in rearrangement.

Earlier studies have indicated that 2,4,5-T genes and IS931 sequences have been recruited from taxonomically distant sources. (Tomasek et al. 1989).
Fig. 5.10 Relative position of RS1100 in pUS1 and pUS105. Open box shows the position of 2,4,5-T genes involved in metabolism of CHQ. Hatched boxes indicate the location of RS1100.
Thus the possibility of phenol degrading genes being recruited from distant microbial species cannot be ruled out. Sangodkar et al., (1988) found no hybridization of xylE gene from P. putida, encoding C230, with AC1100 DNA even under low stringency. However, strain PAA clearly shows the presence of C230 activity. This suggests that the genes of C230 which was silent in strain AC1100 is not homologous to that of P. putida.

Most of the aromatic pathway genes are clustered and strain AC1100 seems to have recruited genes bypassing the requirements for genetic relatedness, leading to gene acquisition from any sources during directed evolution. Danganan et al., (1994) have cloned the 2,4,5-T oxygenase gene which can convert phenoxyacetate to phenol, a suicidal substrate for strain AC1100 because of non utilization. However, the presence of silent gene(s), which is activated during the emergence of PAA indicate the possibility of 2,4,5-T and phenol degrading genes being recruited at the same time during directed evolution. However, it is possible that phenol degrading gene might have been inactivated by insertion sequences like IS931 which on selective pressure in presence of phenol, might transpose adjacent
DNA sequences, especially the 16 kb fragment distal to chq gene(s) leading to gene activation resulting in phenol utilization.