Chapter 3

Cloning of the DDT-degrading genes
5.1 Introduction

In recent years, a number of compounds previously considered non-degradable are also being degraded by microorganisms, suggesting that under selective pressure of environmental pollution, microbes develop the ability to degrade recalcitrant xenobiotics. However, the fact that many pollutants are still persistent in the environment reflects the inadequacy of the current microbial catabolic capacity to deal with such pollutants. That is, the kinetics of process may be much slower than desired. This has stimulated the development of bioremediation technologies which may be applied to many different environmental situations. One of the new developing technologies involves the genetic engineering of natural microorganisms with enhanced or new degradative capabilities for bioaugmentation of select, contaminated environments. Molecular biology offers the tools to optimize the biodegradative capacities of microorganisms, accelerate the evolution of "new" activities, and construct totally "new" pathways through the assemblage of catabolic segments from different microbes. Although the number of genetically engineered microbes (GEMs) for potential use in biodegradation is not large, these recombinant microbes function in microcosms according to their design. The survival and fate of recombinant microbes in different ecological niches under laboratory conditions is similar to what has been observed for the unmodified parental strains. rDNA, both on plasmids and on the host chromosome, is usually stably inherited by GEMs. The potential lateral transfer of rDNA from the GEMs to other microbes is significantly diminished, though not totally inhibited, when rDNA is incorporated on the host chromosome. The behaviour and fate of GEMs can be predicted more accurately through the coupling of regulatory circuits that control the expression of catabolic pathways to killing genes, so that the GEMs survive in polluted environments, but die when the target chemical is eliminated (Ramos et al., 1994).

The formation of DDD from DDT is also a common reaction among soil microorganisms (Guenzi and Beard, 1967). Chacko et al (1966) isolated numerous actinomycetes (Nocardia sp., Streptomyces aureofaciens,
Streptomyces cinnamoneus, Streptomyces viridochromogenes) from soil, which readily degraded DDT to DDD. Wedemeyer (1967) studied DDT metabolic pathway by incubation of proposed intermediates with organisms and examining the products formed. The metabolism of DDT in Aerobacter aerogenes goes in order DDT → DDD → DDMU → DDNU → DDOH → DDA → DBP and direct conversion of DDT to DDE (Wedemeyer, 1967). Reports on the involvement of enzymes in the degradation of DDT indicate the presence of enzymes like dioxygenases, (Nadeau et. al., 1994), dehydrogenases (Bourquin, 1977), oxygenases (Ahmed et. al., 1991). Only a few enzymes have been described in DDT degradative pathway (Singh et. al., 1999). The conversion of DDT to DDD involves a dehydrochlorination step. In our laboratory, an attempt was made to clone, sequence and express the gene responsible for this step.

5.2 Materials

Luria- Bertoni broth and ampicillin were procured from Hi media, Mumbai. Agarose was procured from Sisco Research Laboratories Pvt. Ltd. Taq polymerase dNTPs, and restriction enzymes were procured from Genei, Bangalore. PCR purification kit was purchased from GE Healthcare, UK. Ethidium bromide nitrocellulose membrane and nylon membrane were procured from Sigma Aldrich Chemical Company, MO, US. The designed primers were synthesized by Sigma, MO, US. Cloning kit was procured from Fermentas Lifesciences, EU. DIG kit was obtained from Roche Company, Germany. EDTA was procured from Qualigens Fine Chemicals. All other chemicals used in the study were procured from standard chemical companies.

5.3 Reagents and buffers

5.3.1 Reagents for plasmid isolation

Solution 1: 50 mM Glucose, 25 mM Tris- Cl (pH-8.0), 10 mM EDTA (pH-8.0)

Solution 2: 0.2 N NaOH (freshly prepared from 10 N NaOH), 1%SDS (Prepared freshly before use).
Solution 3:
5.0 M Potassium acetate : 60.0mL
Glacial acetic acid : 11.5mL
Distilled water : 28.5mL

5.3.2 Ampicillin stock solution (100 mg/ ml): Ampicillin was dissolved in distilled water, filter sterilized and stored at -20 °C.

5.3.3 RNAse stock solution: 10 mg of RNAse was dissolved in 1mL of distilled water and boiled for 15 min and used.

5.3.4 CaCl$_2$ solution: 60 mM CaCl$_2$ in Mop’s buffer (pH 6.5)

5.3.5 Luria Bertani broth:
Typtone / peptone : 10.0 g/ L
Yeast extract : 5.0 g/ L
Sodium chloride : 10.0 g/ L
The medium was autoclaved at 15 lbs, 121 °C for 15 min.

5.3.6 Tris- EDTA buffer (pH 8.0)
10 mM Tris Hcl (pH 8.0)
1 mM EDTA (pH 8.0)
pH was adjusted to 8.0, autoclaved and stored.

5.3.7 Tris- acetate EDTA buffer (50X)
Tris buffer : 24.2 g
0.5 M EDTA : 10 mL
Glacial acetic acid : 5.7 mL
Distilled water : 84 mL
pH was adjusted to 8.0, autoclaved and stored.

5.3.8 DNA loading buffer
Bromophenol blue : 0.25%
Xylene Cyanol FF : 0.25%
Glycerol : 30%
This was prepared in double distilled water and stored in aliquots at -20°C.

5.3.9 Alkaline phosphatase buffer
Tris- HCl (pH 9.5) : 100 mM
NaCl : 100 mM
MgCl$_2$ : 50 mM
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1 M Tris (pH 9.5), 5 M NaCl, and 1 M MgCl₂ stocks were prepared in double distilled water, autoclaved and stored at room temperature. Alkaline phosphatase buffer was prepared by adding appropriate amounts of stock solutions, volume made up with double distilled water and stored at room temperature.

5.3.10 Maleic acid buffer
Maleic acid : 100 mM
NaCl : 150 mM

Maleic acid was dissolved in double distilled water containing NaCl. pH was adjusted to 7 with NaOH, autoclaved and stored at room temperature.

5.3.11 Blocking solution: 10% (w/v) BSA was dissolved in maleic acid buffer by stirring and heating, autoclaved and stored at room temperature.

5.3.12 Colour development buffer
BCIP (50 mg/ mL) : 70 μL
NBT (50 mg/ mL) : 70 μL
Alkaline Phosphatase buffer : 10 mL

50 mg/ mL NBT in 70% DMF and 50 mg/ mL BCIP in DMF were prepared and stored at 4 °C protected from light. BCIP may precipitate during storage and should be warmed at room temperature to dissolve.

5.3.13 Church hybridisation buffer
SDS : 7% (w/v)
BSA : 1% (w/v)
EDTA : 1 mM
Na₂PO₄ (pH 7.4) : 0.25 M

1M NaH₂PO₄ and 77.4ml of 1M Na₂HPO₄ were prepared and mixed to produce 1M Na₂PO₄, pH 7.4 stock. For long term storage, this was autoclaved and stored at 4 °C.

The hybridization solution was prepared by dissolving 5 g BSA in ~100ml double distilled water. 125 mL of 1M Na₂PO₄, 175 mL of 20% SDS, and 1 mL of 0.5 M EDTA were added. The volume was made up to 500 mL and stored at room temperature. SDS precipitates out at cool temperature.
When this happens, the hybridization buffer was pre-warmed before use to redissolve the SDS.

5.3.14 20X SSC

<table>
<thead>
<tr>
<th>Substance</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>3 M</td>
</tr>
<tr>
<td>Sodium citrate</td>
<td>0.3 M</td>
</tr>
</tbody>
</table>

175.3 g of NaCl and 88.2 g of sodium citrate were dissolved in ~700 mL of double distilled water. The pH was adjusted to 7.0 with HCl and the volume was made up to 1 L. The solution was autoclaved and stored at room temperature.

5.4 Methods

5.4.1 Microorganisms and culture conditions

All the members of the consortium were grown individually in Luria Bertoni broth for 18 h under shaking conditions (180 rpm). Cells were harvested by centrifuging at 10,000 rpm at 4 °C for 15 min. The supernatant was discarded and pellet was washed with minimal medium (4.3.1), induced with 10 ppm DDT and used for the isolation of genomic and plasmid DNAs.

5.4.2 Isolation of genomic DNA from the bacterial isolates

Genomic DNA was isolated using the method described below:

Suspended a 30 h old induced culture (after centrifugation) in 500 μL lysozyme solution (containing 2mg/mL lysozyme and 50mg/mL heat treated RNase) and incubated at 37 °C for around 30 minutes or till the cells became translucent. To this was added 250 μL of 2% SDS and vortexed gently to mix until the viscosity of the solution decreased noticeably. 250 μL of neutral chloroform solution was added to this and vortexed for 30 seconds, spun for 2 minutes in micro centrifuge and the supernatant was removed leaving the white interface behind. This was repeated twice or till no or very little interface was seen. To this 0.1 volume of 3M sodium acetate (pH 4.8) was added and mixed. This was followed by the addition of 1 volume of isopropanol and mixing. Contents were incubated for 24 h at -20 °C and spun for 5 minutes in the micro centrifuge. Supernatant was poured off. The pellet was redissolved in 500 μL of TE buffer. An agarose gel electrophoresis was carried out to check the genomic DNA.
5.4.3 Isolation of plasmid DNA from the bacterial isolates

Plasmid isolation was done according to Maniatis *et. al.* (1982). Single colonies of appropriate strain grown for 24 h inoculated to 2 mL Luria Bertoni and harvested cells were induced with 10 ppm DDT. The cells were then harvested by centrifuging at 8000 rpm for 5 min and lysed with solution 1. 200 μL of freshly prepared solution 2 was added and mixed. 300 μL of solution 3 was added and centrifuged for 15 min. The supernatant was transferred to a fresh tube and equal volume of phenol- chloroform was added and vortexed thoroughly. The upper aqueous phase was transferred to a fresh tube, equal volume of chloroform was added and centrifuged at 10,000 rpm for 10 min to remove traces of phenol. To the upper aqueous layer, 2 volumes of absolute ethanol was added and kept at -20 °C for precipitation. The tube was centrifuged at 10,000 rpm for 10 min, supernatant was discarded carefully. The pellet was air-dried and dissolved in 20 μL TE buffer. An agarose gel electrophoresis was carried out to check the plasmid DNA. To isolate cloning/ cloned plasmids, ampicillin was added during inoculation. The cells were not induced with DDT.

5.4.4 Designing of the oligonucleotide primers

DDT dehydrochlorinase sequences available in the databank were aligned using Dialign 2.0 software program and primers were designed using Primer 3.0. program and the primer sequence is given in Table 5.1.

Table 5.1: Nucleotide sequences of the primers used for screening DDT- *dhl1* producing bacterial species.

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Primer sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Forward primer <em>Dhl1F</em></td>
<td>CTCGAG GCCGATTG GCC CCGC GATAAC</td>
</tr>
<tr>
<td>Reverse primer <em>Dhl1R</em></td>
<td>AAGCTTAA ATT TG GTT TTC GTT CACGCT</td>
</tr>
</tbody>
</table>

5.4.5 PCR amplification of DDT- *dhl1* gene from *Flavobacterium* sp.

The PCR amplification was carried out with genomic DNA and plasmid DNA with Takara thermocycler unit. The PCR mixture consisted of:

- Sterile deionised water 18.98 μL
- Taq Buffer 2.5 μL
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dNTP mix 0.5 μL
Taq Polymerase 0.3 μL
Forward Primer 1.0 μL
Reverse Primer 1.0 μL
Template DNA/plasmid DNA 1.0 μL

PCR conditions used are as follows:

5.4.5.1 Gradient PCR: A gradient PCR was done with different temperatures from 50-70 °C.

5.4.5.2 Touchdown PCR: A touchdown PCR was done as programmed below:

5.4.5.3 PCR with optimised conditions:

The amplified PCR products were analysed by agarose gel
5.4.6 Analysis of the PCR products:

The gel casting tray was set up with the appropriate comb. Agarose (1%) was dissolved in 1X TAE buffer by heating. It was cooled to 50 to 60 °C and was poured into the gel casting unit. The comb was removed carefully from the solidified gel. The agarose gel was taken in the electrophoresis tank containing 1X electrophoresis buffer. The wells were loaded with the DNA samples mixed with gel loading buffer. Markers (100 bp λ marker) were run along with samples unless otherwise stated. After 3-4 h run at 40 V, the gels were stained with 1% ethidium bromide solution for 10 min and then destained in double distilled water. The DNA was visualised as bands under U. V. transilluminator and documented.

5.4.7 Purification of amplicons of DDT- dhl 1 gene of Flavobacterium sp.

The obtained PCR product was purified by using GE gel purification kit. The bands were excised from the agarose gel by cutting with a clean blade under U.V light and transferred into a 2.0 mL microcentrifuge tube. The following steps were involved in purification of the DNA bands: 10 µL capture buffer type 3 was added for each 10 mg of agarose gel slice. The contents of the tubes were mixed by inverting the tubes and kept in water bath at 60 °C till agarose completely dissolved. GFX™ microspin column was placed in 2.0 mL collection tube provided by the kit. For binding the DNA, the sample was passed through the column and centrifuged at 8,000 rpm for 60 sec. The flow through liquid was discarded and the GFX™ microspin column was placed in new 2.0ml collection tube. The same procedure was repeated until the entire sample was poured. Now 500 µL of wash buffer type1 was added and centrifuged at 8, 000 rpm for 30 sec. The collection tube was discarded and GFX™ microspin column was transferred to clean 1.5 mL DNase free microcentrifuge tube. 10-50µL of elution buffer type 6 (nuclease free water) was added and kept at room temperature for 60 sec. Then, it was centrifuged at 8, 000 rpm for 60 sec to elute the DNA. The obtained purified DNA sample was stored at -20°C and checked by agarose gel electrophoresis.
5.4.8 Cloning of DDT- *dh1* gene of *Flavobacterium* sp.:

5.4.8.1 Ligation of the purified PCR amplicon into pTZ57R/T vector:
Ligation of the purified PCR amplicon was done by using Fermentas kit. To a 2.0 mL microcentrifuge tube, 3 µL vector pTZ57R/T (0.18p mol ends) (Fig. 5.1), 6 µL 5X ligation buffer, 4 µL PCR product (0.54p mol ends), 16 µL nuclease free water and 1 µL T₄ DNA ligase were added and incubated at 4 °C overnight.

Fig. 5.1: Vector map of pTZ57R/T.

5.4.8.2 Transformation of *E. coli* DH5α

5.4.8.2.1 Preparation of competent cells: A single colony of *E. coli* DH5α was inoculated to 50 mL of Luria Bertoni broth and incubated overnight at 37 °C in a shaker. The cells were transferred aseptically to sterile tubes and harvested at 10,000 rpm, 10 min, 4 °C. The above cells were then treated with 10 mL of 60 mM CaCl₂ in MOP’s buffer at a pH 6.5 and vortexed for few minutes. The tubes were centrifuged at 10,000 rpm, 4 °C for 10 min. The
supernatant was discarded and the pellet was resuspended in the 4 mL
solution containing 0.1 M CaCl$_2$. The tubes were kept at 4 °C overnight.

5.4.8.2.2 Transformation of competent cells: Transformation of competent
cells into *E. coli* DH5α was done by using Fermentas transformation kit. 150
µL of overnight stored competent cells were taken and 1.5 mL of C- media
was added. The tubes were incubated at 37 °C for 20 min. The tubes were
centrifuged at 8,000 rpm, 4 °C for 1 min and supernatant was discarded.
The pellet was suspended in 300 µL of T- solution. The tubes were placed
on ice for 5 min. Again, the tubes were centrifuged at 8,000 rpm, 4 °C for 1
min and supernatant was discarded. The pellet was resuspended in 120 µL
of T- solution and kept in ice for 5 min. To 50 µL of above solution, 2.5 µL of
ligation mixture containing 14 ng vector DNA was added and kept in ice for 5
min. The solution was poured to pre-warmed Luria Bertoni media and
incubated at 37 °C overnight.

5.4.9 Selection of transformants

100 µL of transformation mix was plated onto Luria Bertoni agar
plates containing 100 µL/mL ampicillin. The plates were incubated at 37 °C
overnight for the colonies to grow.

5.4.10 Analysis of the transformants

The plasmid profile of the transformed colonies was checked by gel
electrophoresis of plasmids with and without insert.

5.4.11 Isolation of plasmid DNA from transformed colonies

The transformed cells in Luria Bertoni broth were taken in 2 mL micro
centrifuge tube and plasmid isolation was done as described under section
5.4.3. An agarose gel electrophoresis was carried out to check the
transformation.

5.4.12 Sequencing of the cloned genes/transformed plasmids

The transformed plasmid pTZ57R/T containing the DDT-dhl1 insert
was sent for sequencing to Bioserve Biotechnologies Pvt. Ltd.

5.4.13 Sub-cloning of DDT- *dhl1* gene of *Flavobacterium* sp.

5.4.13.1 Isolation of plasmid DNA from transformed colonies: Plasmid
DNA from the transformed colonies was isolated by alkali lysis as described
under section 5.4.3.
5.4.13.2 **Restriction digestion of recombinant plasmids for insert release:** The cloned vector was double digested with *EcoR1* and Hind III for the release of cloned insert. The restriction digestion was carried out as per suppliers’ instructions. After the addition of restriction enzyme along with its buffer, the tubes were incubated at 37 °C for 1 h. The insert release was cross-checked on agarose gel electrophoresis.

5.4.13.3 **Sub-cloning the DDT- *dhl1* insert into *E. coli* DH5α:** The insert DNA was ligated to pUC18 (Fig. 5.2) vector and also pET28a as per the procedure described under 5.4.8.2.1. The cloned plasmid pUC 18 was transformed into *E.coli* DH5α and pET28a (Fig. 5.3) to *E. coli* BL21 according to 5.4.8.2.2. The transformants were selected on Luria Bertoni agar ampicillin plates. The *E. coli* BL21 transformed with pET28a vector were selected with IPTG- X-Gal selection.

**Fig. 5.2: Vector map of pUC18.**
5.4.13.4 Isolation of DDT- dhl1 enzyme

The cells of *E. coli* were grown on Luria Bertoni broth containing ampicillin for 6 h and then were induced with IPTG (0.4 mM). The cells were harvested after 16 h by centrifugation at 10,000 rpm, 4°C for 10 min. Cells were washed with phosphate buffer. The crude extracts of recombinant *E. coli* were prepared by sonication. The supernatant was purified by using HIS- tag purification kit. The eluent was subjected to electrophoresis on a 12% (w/v) SDS-PAGE. The amount of protein was determined by determining O. D. at 280 nm. The enzyme was estimated for activity in microtitre plate. The assay was based on decrease in the pH of a weakly buffered medium containing phenol red as an indicator dye. The colour change from reddish orange to yellow which occur when Cl⁻ is released because of enzyme action on DDT was identified as the enzyme activity. The culture showing dehydrohalogenase activity in micro titre plate was tested for DDT clearance assay on minimal-DDT- agar plate to conform the activity. Spray plates were prepared with 1.5 % agar in minimal medium on petri dishes. The cloned isolate was streaked on to the plate. The surface of the plate was
sprayed with o.1 % DDT as acetone solution. The plates were incubated at 37 °C for 4- 5 days. The formation of clearance surrounding the colonies was used as an indicator of DDT-dehydrohalogenase activity.

5.4.14 Labelling DDT- \textit{dhl1} gene by using DIG kit (digoxigenin- dUTP alkali labile labelling)

The labelling of the DDT- \textit{dhl1} gene was done by using DIG kit. The components of the kit are given in Table 5.2. For the labelling of the DDT-\textit{dhl1} gene of \textit{Pseudomonas putida} \textit{T}4, PCR was carried out as per the procedure 5.4.5.3 and the obtained PCR product was purified by the procedure given in 5.4.7.

The O. D. of the purified product was determined by using spectrophotometer (Schimadzu, Japan) at 260/ 280 nm.

10 ng- 3 µg of DNA was taken and sterile double distilled water was added and the final volume was made upto 15 µL. The DNA was denatured by heating in a boiling bath for 10 min and quickly chilled in ice for 10 min. To this, 2 µL of hexanucleotide mix (vial 5), 2 µL of dNTP labelling mix (vial 6) and 1µL of Klenow enzyme (vial 7) were added, mixed and centrifuged briefly. The reaction vials were incubated at 37 °C for 20 h. After 20 h, the reaction was stopped by adding 2 µL of 0.2 M EDTA (pH 8.0). The labelled probe was stored at -20 °C.

5.4.15 Restriction digestion of genomic DNA

For the restriction digestion of genomic DNA of \textit{Flavobacterium} \textit{T}6, the restriction enzymes used are given in Table 5.3.
Table 5.2: Components of DIG kit.

<table>
<thead>
<tr>
<th>Bottle/cap</th>
<th>Label</th>
<th>Content (including function)</th>
</tr>
</thead>
</table>
| 1          | Unlabelled control DNA | • 20 μL unlabelled control DNA1 [100 μg/ mL]  
• 10 mM Tris-HCl, 1 mM EDTA; pH 8.0  
• Mixture of pBR328 DNA digested separately with Bam H1, Bgl I and Hinf I. The separate digests are combined in a ratio of 2: 3: 3.  
• Sizes of 16 pBR328 fragments: 490, 2176, 1766, 1230, 1033, 653, 394, 298 (2X), 234 (2X), 220 and 154 (2X) bp.  
• Clear solution  
• Controls target in a southern blot.                                                                 |
| 2          | Unlabelled controlled DNA | • 20 μL unlabelled control DNA2  
• [200 μg/ mL]  
• pBR328 DNA that has been linearized with Bam H1  
• Clear solution  
• To practice labelling and to check labelling efficiency.                                                                   |
| 3          | DNA dilution buffer    | • 2 x 1ml DNA dilution buffer  
• [50 μg/ mL herring sperm DNA, in 10 mM Tris-HCl, 1 mM EDTA; pH 8.0 (20 °C)]  
• Clear solution  
• For the dilution steps in the semi- quantitative determination of labelling efficiency.                                      |
| 4          | Labelled control DNA   | • 50μl labelled control DNA  
• Linearized pBR328 DNA, labeled with digoxigenin according to the standard protocol  
• 1μg template DNA and aprox. 250ng digoxigenin- labelled DNA.                                                             |
Table 5.3: Restriction enzymes used for digestion

<table>
<thead>
<tr>
<th>S. no</th>
<th>Type of restriction enzyme</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Sau 3A</td>
</tr>
<tr>
<td>2</td>
<td>Bgl II</td>
</tr>
<tr>
<td>3</td>
<td>Sal I</td>
</tr>
<tr>
<td>4</td>
<td>Bam H I</td>
</tr>
<tr>
<td>5</td>
<td>Xho I</td>
</tr>
<tr>
<td>6</td>
<td>Hind III</td>
</tr>
<tr>
<td>7</td>
<td>Pvu II</td>
</tr>
</tbody>
</table>

- Clear solution
- Estimation of the yield of DIG-labelled DNA.
- 80 µL of 10X conc. Hexanucleotide mix
- [62.5 $A_{260}$ units/ml] random hexanucleotides
- 500 mM Tris-HCl, 100 mM MgCl$_2$, 1 mM Dithioerythritol [DTE], 2 mg/mL BSA; pH7.2
- Clear solution
- Component of the labelling reaction.
- 80 µL of 10X conc. dNTP Labelling mix
- 1 mM dATP, 1 mM dCTP, 1 mM dGTP, 0.65 mM dTTP, 0.35 mM DIG-11-dUTP, alkaline-labile;
  pH7.5 (20°C)]
- Clear solution
- Component of the labelling reaction.
- 40 µL Klenow Enzyme, Labelling Grade
- [2 units/µL DNA Polymerase 1 (Klenow Enzyme, large fragment)]
- Clear solution
- Synthesis of DIG-labelled DNA

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To 6 µL of genomic DNA taken in separate vials, 2 µL each of enzyme buffer and enzyme were added respectively. The vials were kept in the water bath at 37 °C for 24 h.

Agarose gel electrophoresis was carried out for the restriction digested genomic DNA of *Flavobacterium sp. T6*. For the restriction digested genomic DNA, southern blot analysis was carried out.

### 5.4.16 Southern blot analysis of DDT- *dhl1* gene

The conventional transfer method introduced by Southern relies on a gel-sandwich setup. The southern blot was carried out as follows: the gel was placed over a buffer-soaked wick and overlaid with a piece of transfer membrane. A stack of dry paper towels was then placed on the top of the gel sandwich to create a capillary action that “pulls” the buffer from the wick, through the gel and the membrane, and up towards the dry paper towels. Fractioned DNA fragments in the gel will be carried upward by capillary buffer flow and retained on the membrane, thus generating an imprint that is identical to the electrophoresis pattern of the gel. After 18 h of transfer the DNA was then permanently fixed on the membrane by using U. V. cross-linking. Capillary action was usually conducted with a neutral, high salt 20X SSC buffer. The membrane was removed and kept in the hybridization bottle containing 10 mL hybridization buffer (5.3.13) for 1 h at 65 °C. 10 µL of the probe was taken in a fresh eppendorf tube and placed in boiling water for 10 min and immediately chilled on ice. The denatured probe was added to 15 mL fresh hybridization solution and the hybridization was carried out overnight at 65 °C. The hybridization solution was discarded and the membrane was thoroughly rinsed with 10 mL of 2X SSC washing buffer. The washed membrane was transferred into a clean dish containing maleic acid buffer and the membrane was washed for 2-5 min with gentle shaking at room temperature. The membrane was then replaced in the freshly prepared blocking solution and incubated with shaking for 60 min. The blocking solution was discarded and diluted anti-DIG- AP antibody was added and the membrane was incubated at room temperature for 40-60 min with gentle shaking on the rocker. The membrane was then washed with maleic acid buffer for 20 min with shaking and this step was repeated once by using the
fresh washing tray. The membrane was equilibrated in the Alkaline Phosphatase buffer with gentle shaking. The colour development solution was added to the membrane and incubated in the dark at room temperature for 30 min. The colour development was monitored with clear zones.

5.4.17 Production and isolation of antibody

Twenty week old Single comb white leg horn poultry (layers) were immunized with purified DDT- dehydrochlorinase emulsified with Freund’s complete adjuvant. 250 µg of DDT- dehydrochlorinase in PBS buffer (0.5 mL) was mixed well with 0.5 mL of Freund’s complete adjuvant and injected to poultry intramuscularly (at breast muscle). Subsequent immunizations were done with Freund’s incomplete adjuvant and 500 µg of enzyme protein every 15 days for 6 months.

Egg yolk antibody (IgY) was isolated by PEG method. Yolk was separated from egg albumin. 40 mL of phosphate buffer saline (50 mM, pH 7.2) was added for per yolk and stirred for 1 h at room temperature. 10 mL of chloroform/ yolk was added and stirring was continued for another 30 min at room temperature. The precipitate formed was removed by centrifugation. To the supernatant, 14% (w/v) polyethylene glycol 6000 was added and stirred for 90 min at room temperature. The solution was centrifuged at 4°C, 10,000 rpm for 10 min. The precipitate thus obtained (which contains IgY), was dissolved in a known quantity of phosphate buffer saline (50 mM, pH 7.2) and dialyzed against distilled water for 24 h at 4°C to obtain purified antibodies.

5.4.18 Western blot analysis of DDT- dh11 gene

5.4.18.1 SDS-PAGE was done according to Laemmli et. al. (1970).

5.4.18.2 Electrophoretic transfer of SDS-PAGE gel to nitrocellulose membrane: Whatman No 3 filter papers were cut according to the gel size. The nitrocellulose membrane was Cut to the same size of SDS-PAGE gel. The filter papers containing 8 sheets were wetted in transfer solution Glycine, 1.5; Tris 2.9; SDS 0.187; methanol 100 mL and water to 1L) and placed on the transfer unit. Then nitrocellulose membrane was placed on the filter papers and then gel was placed over it. 8 sheets of whatman No 3 were placed over the gel. The current passed trough at the rate of 0.8mA
/cm² for 75 minutes. A part of membrane was stained with amido black (0.02% in distilled water) and rest was taken for western blot.

5.4.18.3 Western blot: SDS-PAGE transferred Nitrocellulose membrane. was blocked with 2% gelatin for 2 hrs and washed with TBST buffer for 10 minutes with three changes. The membrane was incubated with the primary antibody (IgY, 1:5000 in TBST) with shaking at room temperature for 2 hrs. Then the membrane was washed for 10 minutes with three changes in TBST buffer. Then the membrane was incubated with the secondary antibody, Goat anti chicken IgY conjugated to alkaline phosphatase (1:5000 in TBST) with shaking at room temperature for 1 hr followed by washing with TBST for 10 minutes with three changes.

5.4.18.4 Colour development: To 10 mL of alkaline phosphatase buffer pH 9.5 (100 mM NaCl, 5 mM MgCl₂, 100 mM Tris), 66 μL of NBT (25 mg dissolved in 500 μL of 70% dimethyl formamide) and 33 μL of BCIP (25 mg dissolved in 100% dimethyl formamide) were added as substrates. The colour development reaction was stopped by adding 200 μL of 0.5 M EDTA, pH 8.0 in 50 mL 0.9% NaCl or simply by replacing the buffer by water.

5.5 Results

5.5.1 Screening for DDT- dhl 1 producers

Genomic and plasmid DNA from all the ten bacterial isolates were screened by gradient PCR and touch-down PCR for the presence of DDT-dehydrohalogenase primer pairs described in Table 5.1. All the isolates gave positive amplification with genomic DNA (Fig. 5.4) and plasmid DNA (Fig. 5.5). The samples were run on 1% agarose gel. Distinct PCR products of around 750-800 bp were obtained. In our studies, genomic DNA of Flavobacterium species T₆ was chosen for further work.
5.5.2 Optimisation of PCR conditions

To optimise the PCR conditions, gradient PCR between 50-70 °C was carried out. But in all the temperatures, the amplification was not much (Fig. 5.6). The touch-down PCR conditions also did not give better amplification (Fig. 5.6). The PCR done with optimised conditions of denaturation temperature 94 °C for 45 sec, annealing 55 °C for 1.15 min, and extension at 72 °C for 45 sec gave very good amplification (Fig. 5.7).
Large batch amplification and purification of the PCR product

Large batch trails were done to get good quantity of amplified product. The PCR conditions were maintained the same as described under section 5.5.2. A PCR product was run in preparatory agarose gel. The amplified PCR product band was cut and separated from the gel and was purified using PCR product purification kit (GE health care).
5. 5.4 Transformation of competent cells

The PCR product was ligated to pTZ57R/T vector at 4°C for 24 h as per instructions provided along with the kit. *E.coli* DH5α grown overnight in Luria Bertoni broth was treated with CaCl₂ to give competent cells. The ligated pTZ57R/T plasmid vector containing DDT- dehydrohalogenase gene was transformed into competent cells of *E.coli* DH5α.

The transformants were plated on Luria Bertoni agar containing ampicillin (100 µg/mL) (Fig. 5.8). The transformation efficiency was around 50%. A total of about 50 transformant colonies that grew on Luria Bertoni-ampicillin plates were replica plated on to minimal media- DDT- agar ampicillin plates by toothpick method to select DDT dehydrohalogenase clones. 20 of these colonies grew on minimal media- DDT- agar ampicillin plates. These colonies showed good growth in minimal media- DDT- agar ampicillin plates.

**Fig. 5.8: Transformant cells on Luria Bertoni- ampicillin agar.**

5.5.5 Selection of the transformants

The colonies that showed good growth in minimal media- DDT- agar ampicillin plates were then picked up and inoculated to Luria Bertoni broth containing ampicillin (100 µg/mL). The transformants showing maximum growth in the medium were selected for further studies. The transformants from the medium were harvested and the plasmids were isolated from the transformants by alkali lysis method (5.4.3).

The agarose gel electrophoresis of the transformed plasmids run along with untransformed pTZ57R/T plasmid showed that one of the
colonies (E.coli DH- pTZ15) showed an increase in molecular weight indicating the presence of the complete insert (lane 5, Fig. 5.9). The insert in the transformed clone (E.coli DH- pTZ15) was sequenced.

Fig. 5.9: Gel showing the transformed plasmids

The sequence analysis of Flavobacterium species T6 indicated that the protein had 272 residues (Fig. 5.10). This amounted to a reverse translation product of 757 base sequences of most likely codons (Fig. 5.10). The molecular weight of the reverse translated protein has been given as 30,823 with a theoretical pI of 6.42. The aminoacid composition of the reverse translated protein is given in the Table 5.4. The total number of negatively charged residues in the protein (Asp + Glu) are 30 and the number of positively charged residues (Arg + Lys) are 28. The estimated half-life of the protein is shown in the Table 5.4. The restriction analysis of the cloned protein is given in Table 5.5.

Table 5.4: Aminoacid composition of the cloned protein

<table>
<thead>
<tr>
<th>Number of amino acids:</th>
<th>272</th>
</tr>
</thead>
<tbody>
<tr>
<td>Molecular weight:</td>
<td>30823.5</td>
</tr>
<tr>
<td>Theoretical pI:</td>
<td>6.42</td>
</tr>
<tr>
<td><strong>Amino acid composition:</strong></td>
<td>CSV format</td>
</tr>
<tr>
<td>Ala (A) 19</td>
<td>7.0%</td>
</tr>
<tr>
<td>Arg (R) 15</td>
<td>5.5%</td>
</tr>
<tr>
<td>Asn (N) 13</td>
<td>4.8%</td>
</tr>
</tbody>
</table>
Asp (D) 14 5.1%
Cys (C) 9 3.3%
Gln (Q) 8 2.9%
Glu (E) 16 5.9%
Gly (G) 14 5.1%
His (H) 7 2.6%
Ile (I) 23 8.5%
Leu (L) 20 7.4%
Lys (K) 13 4.8%
Met (M) 8 2.9%
Phe (F) 9 3.3%
Pro (P) 12 4.4%
Ser (S) 14 5.1%
Thr (T) 25 9.2%
Trp (W) 11 4.0%
Tyr (Y) 3 1.1%
Val (V) 19 7.0%
Pyl (O) 0 0.0%
Sec (U) 0 0.0%
(B) 0 0.0%
(Z) 0 0.0%
(X) 0 0.0%

**Total number of negatively charged residues (Asp + Glu): 30**

**Total number of positively charged residues (Arg + Lys): 28**

**Atomic composition:**

<table>
<thead>
<tr>
<th>Element</th>
<th>Formula</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carbon</td>
<td>C</td>
<td>1374</td>
</tr>
<tr>
<td>Hydrogen</td>
<td>H</td>
<td>2156</td>
</tr>
<tr>
<td>Nitrogen</td>
<td>N</td>
<td>376</td>
</tr>
<tr>
<td>Oxygen</td>
<td>O</td>
<td>396</td>
</tr>
<tr>
<td>Sulfur</td>
<td>S</td>
<td>17</td>
</tr>
</tbody>
</table>

**Formula:** $C_{1374}H_{2156}N_{376}O_{396}S_{17}$

**Total number of atoms:** 4319
Extinction coefficients:
Extinction coefficients are in units of $M^{-1} \text{cm}^{-1}$, at 280 nm measured in water.

Ext. coefficient: 65470
Abs 0.1% (=1 g/l) 2.124, assuming all pairs of Cys residues form cystines
Ext. coefficient: 64970
Abs 0.1% (=1 g/l) 2.108, assuming all Cys residues are reduced

Estimated half-life:
The N-terminal of the sequence considered is A (Ala).
The estimated half-life is: 4.4 hours (mammalian reticulocytes, in vitro).
>20 hours (yeast, in vivo).
>10 hours (Escherichia coli, in vivo).

Instability index:
The instability index (II) is computed to be 34.09
This classifies the protein as stable.

Aliphatic index: 88.90

Grand average of hydropathicity (GRAVY): -0.110

Fig. 5.10: DDT- dhl 1 gene sequence

```
+1 Ala Ile Gly Arg Val His Asn Leu Asp Ile Lys Ile Ile Leu Met t Asn
1 GCC ATC GGC CGC GTG CAC ATC AAG ATC ATC CTG ATG AAC
     Asn Glu
     AAC CAG
     Leu Met Phe Asn Glu His Ille Val Ala Ser Ala Tyr Pro Tyr Gln Thr
85 CTG ATG TTC AAC GAA CAC ATC GTG GCC AGC GCC TAC CCG TAC CAG ACC
     Asp Thr
     GAC ACC
     Asp Leu Asn Lys Asp Ser Asp Pro His Ala Ala Leu Gln Ala Ala Ile
169 GAC CTG AAC AAG GAC AGC GAC CCG CAC GCC GCC CTG CAG GCC GCC ATC
     Glu Arg
     GAA CGC
     Asp Val Ser Glu Lys Val Trp Pro Met Val Leu Pro Gly Asp Ala Asn
253 GAC GTG AGC GAA AAG GTG TGG CCG ATG GTG CTG CCG GCC GAG GCC AAC
     Ile Asp Val
     ATC GAC ATG
     Arg Arg Thr Glu Ser Trp Lys Lys Ile Ile Ser Leu Ser Leu Trp Asn
337 CGC CGC ACC GAA AGC TGG AAG AAG ATC ATC AGC CTG AGC CTG TGG AAC
     Trp Leu
     TGG CTG
     His Thr Phe Ala Val Cys Leu Pro Ala Gly Arg Leu Thr Trp Thr Val
```
Table 5.5: Restriction analysis of the cloned protein.

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<th>Name</th>
<th>Pattern</th>
<th>Length</th>
<th>Overhang</th>
<th>Number of cut sites</th>
<th>Cut position(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ApaI</td>
<td>gggccc</td>
<td>6</td>
<td>3'</td>
<td>1</td>
<td>229</td>
</tr>
<tr>
<td>Ball</td>
<td>tggccca</td>
<td>6</td>
<td>Blunt</td>
<td>1</td>
<td>109</td>
</tr>
<tr>
<td>BglII</td>
<td>gccnnnnnggc</td>
<td>11</td>
<td>3’</td>
<td>1</td>
<td>205</td>
</tr>
<tr>
<td>EcoRII</td>
<td>ccwgg</td>
<td>5</td>
<td>5’</td>
<td>6</td>
<td>20, 50, 56, 457, 521, 542</td>
</tr>
<tr>
<td>HpaII</td>
<td>ccgg</td>
<td>4</td>
<td>5’</td>
<td>9</td>
<td>223, 286, 323, 403, 442, 446, 670,</td>
</tr>
<tr>
<td>Kpn2kl</td>
<td>ccngg</td>
<td>5</td>
<td>5’</td>
<td>11</td>
<td>20, 50, 56, 221, 222, 285, 401, 457, 521, 542, 669</td>
</tr>
</tbody>
</table>
The ExPASSy blast results for finding out the homology of DDT-\(dhl\) 1 gene with \textit{Pseudomonas} species genome indicated (Fig. 5.11) that the sequence had 24-26% identity with \textit{Ps. putida} genome. The DDT-\(dhl\) 1 gene showed homology with acetolactate synthase gene sequence. The southern blot analysis of genomic DNA from \textit{Flavobacterium} species for the DDT-\(dhl\) 1 probe was done. The genomic DNA of \textit{Flavobacterium} species yielded positive hybridisation. More than 1 hybridising bands were observed for few restriction enzymes digested. \textit{EcoR1} yielded around 5 bands whereas others yielded only 2 bands. The cloned insert was sub-cloned into pUC18 and pET28a vectors. The expressed protein was analysed for enzyme activity, product of enzyme action and western blot analysis. The colorimetric assay is shown in Fig. 5.12. The enzyme exhibited colour change of the dye
because of chloride release. The DDT-dehalogenase positive colonies also exhibited a clearing zone on minimal medium-DDT-agar plates (Fig. 5.13). DDT precipitation disappeared in 5 days because of the degradation of the compound. The western blot analysis gave positive colour with the cloned protein (Fig. 5.14). The product of analysis of the enzyme action indicated formation of DDD as identified by GC-MS analysis (Fig. 5.15).

**Fig. 5.11: ExPASSy blast results**

![ExPASSy blast results](image)

**Alignments**

[OrderedLocusNames=Pput_2633;] [Pseudomonas putida (strain F1 / ATCC 700007)]

Score = 30.0 bits (66), Expect = 0.13

Identities = 15/61 (24%), Positives = 28/61 (45%), Gaps = 12/61 (19%)

Query: 6 RPGPCLIHALIDVSEKVWPMVLPDANIDMTANHGGKRRESW----KKIISLSLN 59

```
  +P  P  I++L  +++P  +  +  T+NH  ++RR
W  ++  SLW
```

Sbjct: 408 QPAPLFINSLF------
KVIIPDQHLLVALYTSNHRERRRRKAGWGAAGLAALVLCSLWG 461

Query: 60 W 60

```
  W
```

Sbjct: 462 W 462
[OrderedLocusNames=Pput_0611;] [Pseudomonas putida (strain F1 / ATCC 700007)]

Score = 28.1 bits (61), Expect = 0.51

Identities = 13/50 (26%), Positives = 27/50 (54%), Gaps = 4/50 (8%)

Query: 11
LIHALIDVSEKVVPMVLPGDANIDMVTANHGKKKRTESWKIIISLSLWNW
60

Sbjct: 24 VLGSFLWLAIRWPLPGDLARVLAEDG----TPLWRFAADVWRY 69

SubName: Full=Putative uncharacterized protein;

[OrderedLocusNames=Pput_4099;] [Pseudomonas putida (strain F1 / ATCC 700007)]

160 AA

Score = 25.8 bits (55), Expect = 2.5

Identities = 12/44 (27%), Positives = 22/44 (50%), Gaps = 5/44 (11%)

Query: 92 RCRDRITAISGCW----TKNRMDGKTARWSVRWKWKMCRTTT
131

Sbjct: 39 RYRTVPTLVNVSMFTATEAKLFDFG-WRKWGKADWFCLPLK 81
Fig 5.12: Colorimetric assay of DDT dehydrohalogenase enzyme

Red colour: Control
Yellow: DDT-dehydrohalogenase positive

Fig 5.13: DDT-dehydrohalogenase positive plates
Fig. 5.14: Western blot hybridization of DDT-dhl1 protein of *Flavobacterium* sp. probed with antibody raised against DDT-dehydrohalogenase enzyme

Fig. 5.15: GC-MS pattern

B. DDD
The DDT dehydrohalogenase gene from the clone *E.coli* DH- pTZ15 was recovered by restriction digestion and was ligated to restriction digested pUC18 cloning vector. The transformants were selected as given above. The insert was recovered from the cloned vector by restriction digestion and this was transformed into pET28a vector. This transformed plasmid vector was cloned into *E.coli* BL21.

The purified PCR product of *Pseudomonas putida* T5 was labeled using non radio active nucleic acid labelling by DIG- labelling kit system according to users guide as described above. The genomic DNA of *Flavobacterium* species was restriction digested with Sau 3A, Bgl II, Sal I, Bam HI, Xho I, Hind III, Pvu II restriction enzymes using appropriate buffers and incubating for 16hrs at 37 °C. The restricted fragments were separated on 1% agarose gel and transferred on to Nylon membrane. The DDT-DHL probes were allowed to hybridize with the denatured DNA on the nylon membrane. The southern hybridization of genomic DNA from the strain *Flavobacterium* species revealed the hybridizing DNA fragments for the DDT- DHL probes tested. More than one hybridizing band was observed for few restriction enzymes used for digestion (Fig. 5.16). These results indicated that presence of more than one band may be due to the presence of more than one copy of the gene.

Southern blot hybridization with plasmids isolated from the native bacteria *Flavobacterium* species and amplified with PCR, revealed homology with DDT degrading gene i.e; the nylon blot of plasmid DNA showed hybridization with the probe DNA of DDT-DHL. This indicated that the DDT-Dehydrohalogenase gene might be present either as partial or complete gene on the native plasmid (Fig. 5.17).
Fig. 5.16: Southern blot hybridization of genomic DNA of *Flavobacterium* sp. probed with DIG labelled T$_5$ probe

Fig. 5.17: Southern blot hybridization of plasmid DNA of *Flavobacterium* sp. probed with DIG labelled T$_5$ probe
5.6 Discussion

The recalcitrance of many synthetic chemicals to biodegradation is mainly due to the lack of enzymes that can carry out critical steps in the catabolic pathway. This especially holds good for low-molecular weight halogenated compounds. These xenobiotic chemicals are less water soluble and less bio-available. Theoretically, these could be converted by short metabolic routes to intermediates that support cellular growth under aerobic conditions. Yet, no organisms have been found that oxidatively degrade and use these important environmental chemicals as a carbon source. Attempts to obtain enrichments or pure cultures that aerobically grow on these chemicals have met no success. However, some other halogenated chemicals are easily biodegradable, and cultures that utilize chloroacetate, 2-chloropropionate and 1-chlorobutane can be readily enriched from almost any soil sample (Leisinger, 1996; van Agteren et al., 1998). For still other compounds, degradative organisms have been isolated, but only after prolonged adaptation due to pre-exposure to halogenated chemicals in the environment. In our studies also, a DDT-degrading microbial consortium was isolated after a long enrichment of the DDT-contaminated soil. All the individual isolates i.e. ten bacterial strains that constituted the consortium, were found to be necessary for the complete degradation of DDT. These individual isolates were found to act synergistically during degradation of DDT.

With halogenated compounds, an obvious critical step in a potential biodegradation pathway is dehalogenation. Biochemical research with organisms that grow on halogenated compounds has shown that a broad range of dehalogenases exists, both for aliphatic and aromatic compounds. The first enzyme that is responsible in the dehalogenation of DDT to DDD has been identified as DDT-dehydrodehalogenase (DDT-\textit{dhl} 1). So far, there are hardly any reports on bacterial DDT-dehydrodehalogenase (DDT-\textit{dhl} 1) enzyme and genes encoding the enzyme. An attempt was made in our laboratory to isolate and clone bacterial DDT-dehydrodehalogenase (DDT-\textit{dhl} 1). \textit{Flavobacterium} sp. T6 was chosen after preliminary screening by gradient PCR and touchdown PCR. The PCR amplified gene product was
cloned and sequenced. The gene showed sequence similarity of 93 % to Acetolactate synthase of *Serratia sp.*. There are many xenobiotic degrading enzymes which have shown such sequence similarities to other family of enzymes. An interesting type of aliphatic dehalogenase is the enzyme (LinA) that is responsible for the first step in the bacterial degradation of lindane (γ-hexachlorocyclohexane) where HCl was eliminated, converting the substrate to pentachlorocyclohexene (Nagata *et. al.*, 2001; Trantirek *et. al.*, 2001). The structure has not been solved, but a mechanism was predicted on the basis of the stereochemistry of the reaction and low but significant sequence similarity to scytalone dehydratase. Similarly, hydrolytic dehalogenase degrading chlorobenzoate (CbzA) (Benning *et. al.*, 1998) has been shown to belong to the enoyl hydratase superfamily. A specific hydrolytic dehalogenase (AtzA), involved in the bacterial degradation of atrazine has been shown to be related to melamine deaminase (TriA) (Seffernicbelongk *et. al.*, 2001). Dichloromethane dehalogenase (DcmA) catalysing the conversion of dichloromethane to formaldehyde in a glutathione-dependent reaction and another group of dehalogenating proteins chloroacrylic acid dehalogenases (CaaD), which are present in bacteria that degrade the nematocide 1,3-dichloropropene have been found to belong to the tautomerase superfamily of proteins. Halohydrin dehalogenases belong to short-chain dehydrogenase reductase (SDR) superfamily of proteins (de Jong *et. al.*, 2003). Alkane hydrolase (AlkB) belongs to a large superfamily of proteins that also includes non-haem integral membrane desaturases, epoxidases, acetylenases, conjugases, ketolases, decarbonylases and methyl oxidases. Atrazine chlorohydrolase (AtzA) belongs to the amidohydrolase superfamily. Other members of the amidohydrolase superfamily are triazine deaminase, hydantoinase, melamine deaminase, cytosine deaminase and phosphotriesterase. *Rhodococcus* haloalkane dehalogenase (DhaA), the dehalogenase gene was preceded by the same invertase gene sequence and a regulatory gene, and on the downstream side an alcohol dehydrogenase and an aldehyde dehydrogenase encoding gene (Poelarends *et. al.*, 2000b).
Analysis of the genetic organization of biodegradation pathways provides insight into the genetic processes that led to their evolution. It appears that catabolic genes for xenobiotic compounds are often associated with transposable elements and insertion sequences. They are also frequently located on transmissible plasmids. One striking example of a mobile element that has assisted catabolic genes in their dissemination is IS 1071. This insertion element flanks the haloacetate dehalogenase gene dehH2 on plasmid pUO1 in Moraxella sp. strain B (Kawasaki et. al., 1992), the haloalkane dehalogenase gene dhaA on the chromosome in P. pavonaceae 170 (Poelarends et. al., 2000a), the atrazine degradative genes atzA atzB and atzC on plasmid pADP-1 in Pseudomonas sp. ADP (Wackett, 2004), the aniline degradative genes on plasmid pTDN1 in Pseudomonas putida UCC22 (Fukumori and Saint, 1997), and presumably also the p-sulfobenzoate degradative genes on plasmids pTSA and pPSB in Comamonas testosterone strains T-2 and PSB-4 respectively (Junker and Cook, 1997). These observations clearly indicate that gene mobilization between and within replicons is an important process during genetic adaptation. It also suggests that genes that are involved in biodegradation of xenobiotics were recruited from a ‘pre-industrial’ gene pool by integration, transposition, homologous recombination and mobilization. Association of dehalogenase sequences with mobile genetic elements has also been observed in other cases, viz. with haloacetate dehalogenases (Slater et. al., 1985; Thomas et. al., 1992; van der Ploeg et. al., 1995), with dichloromethane dehalogenase (Schmid- Appert et. al., 1997), and with γ-hexachlorocyclohexane dehalogenase (Dogra et. al., 2004). In our studies also, the native plasmid harboured by the bacteria Flavobacterium sp. showed a positive amplification with primers pairs of DDT-dehydrohalogenase gene. The Southern blot analysis also supported the findings where hybridization was observed with the PCR amplified DDT- dhl 1 product of the plasmid and the DDT- dhl 1 probe.

It has become clear that most dehalogenases belong to protein superfamilies that harbour both dehalogenases and proteins that carry out completely different reactions (de Jong and Dijkstra, 2003). Hisano et. al.,
(1996) and Ridder et al., (1999) defined the haloalkane dehalogenases, belonging to HAD superfamily of hydrolases. Possessing a α/β- hydrolase fold main domain. The α/β- hydrolase structural fold is shown also to be found in lipases, acetylcholinesterases, esterases, lactonases, epoxide hydrolases and others, showing that the haloalkane dehalogenases belong to a protein superfamily of which the members carry out diverse reactions, mostly with non-halogenated compounds.

Less is known about the origin of the structural genes that encode critical enzymes, such as dehalogenases, and the degree of divergence that occurred during evolution of the current sequences. Theoretically, it is possible that a current gene for a specific critical (dehalogenase) reaction was already present in the pre-industrial gene pool. Alternatively, there could be a short evolutionary pathway that led from an unknown pre-existing gene to the gene as we currently find it in a biodegradation pathway. It has even been suggested that a new sequence for an enzyme acting on a synthetic compound could evolve through the activation of an unused alternative open reading frame of a pre-existing internal repetitive coding sequence (Ohno, 1984). Here it should be noted that the similarity of a dehalogenase to members of an enzyme superfamily that catalyse other reactions generally does not provide information about the process of adaptation to xenobiotic compounds. The level of sequence similarity that exists between a dehalogenase and other proteins in a phylogenetic family is usually less than 50%. Therefore, the time of divergence should be much earlier than a century ago, and the process of divergence thus cannot be related to the introduction of industrial chemicals into the environment. If the dehalogenases and other critical enzymes that occur in catabolic pathways have undergone recent mutations, there should be closely related sequences in nature that differ from the current enzymes by only a few mutations. No such primitive dehalogenase has yet been detected, with the notable exception of TriA, the enzyme that dehalogenates the herbicide atrazine. Another issue is the function of the pre-industrial dehalogenase or dehalogenase-like sequences from which the current catabolic systems with their activated and mobilized genes originate. The original genes may have
been involved in the dehalogenation of naturally occurring halogenated compounds, of which there are many (Gribble, 1998). Such proteins may fortuitously also have been active with a xenobiotic halogenated substrate, just because of their lack of substrate specificity. Alternatively, the evolutionary precursor of a dehalogenase may have catalysed a different reaction that has some mechanistic similarity to dehalogenation, in which case the original enzyme may or may not have shown some dehalogenation activity due to catalytic promiscuity. Finally, the precursor genes for dehalogenases may have been silent or cryptic genes, with no clear function for the pre-industrial host (Hall et al., 1983). In all cases, the gene could have become functional in a dehalogenation pathway as a result of the fortuitous ability to catalyse dehalogenation of a xenobiotic compound, possibly after acquisition of some mutations.

One way to obtain information about the evolutionary origin of dehalogenase genes is to compare the dehalogenase sequences that have been detected in different bacterial cultures. If closely related sequences are present, this would make it possible to identify sequence differences and to determine the effect of the mutations on substrate selectivity. Currently, the whole sequence of more than 300 bacterial genomes is available. If we find closely related sequences in these databases, they could define evolutionary ancestors of the current dehalogenases.

When searching for putative alkane hydroxylase genes in environmental DNA (Venter et al., 2004), a large number of alkB and alkM homologues were again detected, including two sequences that were 82% similar to alkB.

Even though the same dehalogenase sequences are detected in organisms that are isolated in different geographical areas, and in some cases even on different substrates, they are not the most abundant dehalogenase sequences identified in whole genome sequencing projects and massive random sequencing. Thus, it appears that enrichment techniques explore a different segment of sequence space than massive sequencing of environmental DNA. The presence of large numbers of unexplored functional sequences in genomic databases suggests that the
Biotransformation scope of microbial systems has an enormous potential for further growth.

5.7 Conclusions

Bacterial dehalogenases catalyse the cleavage of carbon-halogen bonds, which is a key step in aerobic mineralization pathways of many halogenated compounds that occur as environmental pollutants. DDT-dehydrohalogenase catalyses the dehydrohalogenation of DDT to DDD with the removal one HCl. The gene responsible for the dehalogenation was cloned and sequenced. The gene sequence showed similarity to acetolactate synthase gene. There is a broad range of dehalogenases, which can be classified in different protein superfamilies and have fundamentally different catalytic mechanisms. Identical dehalogenases have repeatedly been detected in organisms that were isolated at different geographical locations, indicating that only a restricted number of sequences are used for a certain dehalogenation reaction in organohalogen-utilizing organisms.

One of the striking observations concerning the distribution and evolution of key catabolic genes is that identical sequences have repeatedly been detected in organisms that are enriched on xenobiotic halogenated substrates as a carbon source. Probably, the number of solutions that nature has found to degrade these compounds is very small, and horizontal distribution occurs faster than generation of new pathways. Indeed, the dehalogenase genes are often associated with integrase genes, invertase genes, or insertion elements, and they are usually localized on mobile plasmids.

It is likely that ongoing genetic adaptation, with the recruitment of silent sequences into functional catabolic routes and evolution of substrate range by mutations in structural genes, will further enhance the catabolic potential of bacteria toward synthetic organohalogenes and ultimately contribute to cleansing the environment of these toxic and recalcitrant chemicals.
5.8 Bibliography


Cloning of DDT degrading genes


