Chapter 7: Designing of Molecular Probe for Characterization of Catabolic Gene Cluster Involved in Degradation of Dibenzoofuran
Designing of Molecular Probe for Characterization of Catabolic Gene Clusture Involved in Degradation of Dibenzofuran

7.1 INTRODUCTION

Many aromatic compounds are metabolized by bacteria, and the metabolism is the result of the traits present in bacteria which may be due to adaptive activities. It may be acquired also by horizontal gene transfer (HGT) from the other members of microbial community (Top and Springael, 2003). The genes involved in catabolic pathways are often found clustered i.e. having similar gene organization and composition irrespective of phylogenetic position of the bacteria (Egland et al., 1997; Furukawa, 1994; Harayama et al., 1992; Poelarends et al., 2000; Van der Meer et al., 1992; Williams et al., 1994).

This catabolic potency sometimes distributed throughout the community by HGT with the help of mobile elements like transposons, plasmid through conjugation (Tan, 1999; Tsuda, 1999; Harayama et al., 1989). So far, varieties of catabolic clusters have been reported in different strains. However, the diversity among the catabolic clusters prevails due to the internal rearrangements by deletion, insertion, repetition, inversion for gaining the catabolic potency against any compound (Hallet et al., 1997; Wyndham et al., 1997; Yen and Serdar, 1988).

There are several reports of the entire catabolic pathways for the persistent organic pollutants such as naphthalene, toluene, and biphenyl. Probably, such genetic structures developed after a long exposure to corresponding compounds. However, the catabolic genes encoded for recalcitrant molecules seem to be scattered throughout the genome. One possible explanation could be that the degree of the evolution in different genetic structure could be different during the time of exposure. Thus the catabolic genes are important in revealing the evolution stage of gene cluster (Nojiri et al., 2001).
Dibenzofuran is oxygen containing heterocyclic aromatic compound, precursor of dioxin and known to have toxic activities due to its planner structure, it is a recalcitrant molecule. *Pseudomonas* sp. strain CA10 is a microorganism having the ability to utilize dibenzofuran as a sole carbon source. Strain CA10 degrades dibenzofuran to anthranilate and 2-hydroxypenta-2, 4-dienoate by angular dioxygenation, meta-cleavage and hydrolysis. Anthranilate is attacked by anthranilate dioxygenase at its 1, 2 positions and yield catechol, which is degraded by the β-ketoadipate pathway later. Whereas, 2-hydroxypenta-2, 4-dienoate is converted to a tricarboxylic acid (TCA) cycle intermediate by the three additional conversions called the meta-cleavage pathway (Ouchiyama *et al.*, 1993; Bressler and Fedorak, 2000). In the present work, we have cloned the anthranilate 1, 2 dioxygenase (β) component based on MALDI-TOF data and synthesized its recombinant form which can be very useful for tracking the entire gene cluster.

### 7.2 MATERIALS AND METHODS

#### 7.2.1 Bacterial strains and plasmids

The bacterial strains used in this study are listed. *Pseudomonas aeruginosa* wild-type strain ISTDF1 was isolated from pulp-bleaching wastewater as described in previous chapters. *Escherichia coli* BL21 were used as host cells and for plasmid vector (pQE30) (Quigen Inc.) was used for cloning and expression studies.

#### 7.2.2 Media and growth conditions

*E. coli* BL21 were routinely grown in Luria-Bertani (LB) medium at 37°C, containing kanamycin (25 mg/liter) and ampicillin (100 mg/liter) respectively.

#### 7.2.3 Agarose gel electrophoresis

Agarose gel electrophoresis was performed at 70 volt. Agarose gel (Sigma-Aldrich) were boiled in 0.5X TBE buffer (45mM Tris-borate, 1mM
EDTA at pH 8.3) maintaining 0.8% agarose and EtBr (0.5μg/ml) and casted in horizontal electrophoresis (Owl, Inc).

7.2.4 Primer Designing, PCR and Sequencing

The degenerate primers were designed based on MALDI-TOF data. Genomic DNA from Pseudomonas aeruginosa ISTDF1 strain was isolated with the Genome DNA Kit (Qiagen Inc., USA) as described by the manufacturer. The sequence was selectively amplified from genomic DNA by using polymerase chain reaction (PCR) with oligonucleotide -degenerate primers PCR in a DNA Thermal Cycler (Applied Biosystem Inc, USA) under the following conditions: 10–20 ng template DNA, 5 μl 10x reaction buffer, 2·5 U Taq DNA polymerase, 1 μM forward primer, 1 μM reverse primer, 20 μM of each dNTP and H2O combined in a total volume of 50 μl.

The tubes were incubated at 94°C for 5 min and then subjected to the following thermal cycling programme: denaturation at 94°C for 1 min, primer annealing at 60°C for 1 min, and chain extension at 72°C for 30 second with an additional extension time of for a total of 30 cycles. The amplified DNA was purified using Qiaquick PCR Purification Kit (Qiagen), adjusted to 200 ng μl⁻¹, and sent to the M/S Bangalore Genei, India for sequencing. Sequenced data was compared and analyzed by existing database of GeneBank, National Center for Biotechnology Information. Phylogenetic tree was drawn on the basis of the sequences. Bootstrap consensus tree (1000 copies) was drawn by multiple sequence alignment with Neighbor-Joining method using software Mega, version 4.0 with related sequences in different species of bacteria.

7.2.5 DNA Cloning

All of the recombinant DNA methods, used to construct the plasmids to study the cloned fragments, had been done as described previously Sambrook et al., 1989. For cloning, expression vector pQE30 (Quigen Inc, USA) was used. Cloning protocol was followed as per suggestion of manufacturer. Nucleotide sequencing was done commercially from Bangalore
Genie, India. The nucleotide and deduced amino acid sequences were analyzed with DNAMAN 4.0 software (Lynnon Biosoft, Jerusalem), and a similarity search was carried out with the NCBI database.

7.2.6 Induction, Expression and SDS-PAGE

An overnight culture of the E. coli strain BL21 harboring the plasmid pQE30 with ~0.25kb insert was inoculated in LB fresh medium. Isopropyl-b-D-thiogalactopyranoside (IPTG) was added to create a final concentration of 1 mM when the OD_{595} of the culture reached to 0.1., and the cells were suspended in lysis buffer. Sample were subjected to Ni-NTA column for enrichment of histidine tagged recombinant enzyme as per suggested by manufacturer (Bangalore Genie, India). The eluent was re-eluted from the column. After taking OD at 280 nm the, the eluted samples were boiled for four min, and each sample was loaded in 12% polyacrylamide gel electrophoresis (PAGE). After electrophoresis, the gel was stained with staining buffer having Coomassie Brilliant blue (CBB) as described by Cook and Bunz, 1993. The recombinant band was identified as anthranilate 1,2 dioxygenase (β subunit). The further conformation was done by MALDI-TOF.

7.3 RESULTS

All results were shown below with the relevant details.

7.3.1 MALDI-TOF analysis of anthranilate 1, 2 dioxygenase β subunit

Band was excised from point 'O' as shown in Figure 7.1. MALDI-TOF analysis was performed as shown in Figure 7.2. The fragment was identified as anthranilate 1, 2 dioxygenase β subunit having molecular weight (~19 k Da). This data is further confirmed by MS/MS of MALDI data as shown in Figure 7.3. It shows very good homology with anthranilate dioxygenase. It is a very important enzyme in dibenzofuran degradation. It degrades salicylate to form catechol in lower pathway.
Figure 7.1: Anthranilate 1, 2 Dioxygenase (β) is indicated as 'O' in the encircled band 12% SDS-PAGE.

Figure 7.2: MALDI-TOF of the Anthranilate 1, 2 Dioxygenase (β)
Figure 7.3: MS/MS of MALDI-TOF data for the Anthranilate 1, 2 dioxygenase (β subunit).

Anthranilate 1, 2-dioxygenase, β subunit, putative- Pseudomonas fluorescens (strain Pf-5/ ATCC BAA-477).

MNQILQYQIEQFFYRKSELCDADWDAYLQLFAVDSEFHLQWDEHVVTRDPKREMISLLYANRSGLEDLVRLRTGKAAS
MPRTLHMVSNLRIAEQDNAIGLLRVNLWHTLFYRLATSEEFYGHATYDLKQGDSWLLIQKHSVLLNMTINSVLDYHL
7.3.2 Primer designing

Based on the anthranilate 1, 2 dioxygenase (β) data, degenerate primers were designed. Considering the complimentarily and self-complimentarily constants, primers were designed between the 6th amino acid to 117th amino acids of this enzyme sequence by DNAMAN software.

Abbreviation to represent ambiguity:
R = A or G, Y = C or T, H = A, C or T, N = A, C, G or T

1 METAsnAlaGlnLeuGlnTyrGlnIleGluGlnPhePheTyrArgLysSerGluLeuCys
   ATGAAYGCNCACRNCAARTYCARATHGARCARTTTYTAYCGNAARTCNGARCTNTGY
   TTR AGR AGR T

21 AspAlaGlnAspTrpAspAlaTyrLeuGlnLeuGlnTyrArgLysHisLeu
   GAYGCNCARGAYTGAGAYGCTNTCRTNATYGCNGTGAYTNGARTTYCACTN
   TTR TTR AGR AGR T

41 ProGlnTrpAspSerGluHisValTyrThrArgProLysArgGluMETSerLeuIle
   CCNCARTGGGAYTCNGACAYGTNTAYACNCNGAYCNCNGARATGCNTCNATH
   AGR AGR AGR AGR

61 TyrTyrAlaAsnArgSerGlyLeuGluAspArgValLysArgThrGlyLysAla
   TAYTAYGCNAYCNCNGGNTNARGAYCNGNTNTYGCNGTNGCNACNGAARGCN
   AGRAGY AGRAGY AGR AGR AGR TTR AGR AGR AGR

81 AlaSerThrIleProMETProArgThrLeuHisMETValSerAsnLeuArgIleAlaGlu
   GCNACNACITHCNCATGGCNCNGNAACNCTCAYATGGTNTCNAAYCNCNGNATHGCNGAR
   AGRAGY TTR AGR AGR AGR

101 GlyAspAsnGlyLeuLeuArgValArgLeuAsnTrpHisThrLeuPheTyrArgLeuAla
   GGNGAYAAYNCNGNTNCGNTNCAAYYGCAYACNTNTTAYACNTCNCTNGC
   TTRTRAGR AGR AGR AGR AGR

121 ThrSerGluPheTyrGlyHisAlaThrTyrAspLeuLysProGlnGlyAspSerTrp
   ACNACNACRARTYIAYGNCAYGCNACNTAYAGCNAARCNNCCARGNGAYTCNGTGG
   AGR TTR AGR AGR AGR

141 LeuIleGlnArgLysHisValLeuLeuLeuAsnThrIleAsnSerValLeuAspPhe
   CTNACNACNCACAYTNGTNCNTCNAAYGACNATHAAYTNGTNGAYTTY
   TTR AGR TTR AGR AGR

161 TyrHis

481 TAYCAY

Figure 7.4: Reverse Translation of Anthranilate 1, 2 Dioxygenase (β) sequence obtained from MALDI-TOF and appropriate regions selected for designing degenerate primer showing in box.

7.3.3 PCR amplification

PCR amplified was found to be around 250 base pairs (bp) which was cloned in expression vector pQE30-UA.
7.3.4 Cloning and sequencing of Anthranilate 1, 2 Dioxygenase (β) gene

Cloned vector was amplified and sent for sequencing. The blast results confirmed its close homology with anthranilate dioxygenase. With this, it was also showing a close homology with a ring hydroxylating enzyme. Sequencing was done from Bangalore Genei Inc., India.

Sequence quality of cloned PCR amplified with degenerate primer was quiet good as shown in Figure 7.8. The sequence was identified by subtracting the plasmid sequence 6. As shown in 9, sequence color with the orange and green color is vector sequence whereas sequence written in black is, cloned sequence. In Figure 7.7, no amplification was found from uncloned plasmid whereas as it was found in cloned plasmid. It was double digested with HindIII and BamH1 which give rise to ~250 sequences as shown in lane 11 and 12 of Figure 7.7.
Figure 7.6: Plasmid map of 3.5 kb pQE30-UA as per given by manufacturer. In left side of plasmid plan, sequence arrangement and site of cloning where PCR product are cloned are shown.
Figure 7.7: DNA Cloning of ant (β) has been shown in plasmid vector pQE30. In Lane M, 1kb Marker; lane 1, 2 and 3, PCR amplified from without cloned plasmid, cloned plasmid and strain C respectively; in lane 4 and 5 only pQE30 (control) are loaded; in lane 6, 7, 8, 9, 10 plasmid isolated from positive clone; lane 11 and 12 double digested plasmid with HindIII and BamH1 showing release of ant β from the plasmid from the clone used in lane 9 and lane 10 respectively.

Figure 7.8: Sequencing quality of cloned DNA fragment in plasmid vector pQE30.
Figure 7.9: Sequences reflected in black letters are the sequence of cloned DNA fragment in plasmid vector pQE30-UA.

7.3.5 Phylogenetic placement

Phylogenetic placement of the cloned sequence was done which is identified as anthranilate 1, 2 dioxygenase (β subunit). However, this sequence was showing flanking regions of carbazole catabolic cluster. The sequencing result was also shown to have close homology with benzoate 1, 2 dioxygenase as shown in Figure 7.10.
Figure 7.10: Phylogenetic placement of the cloned sequence which is identified as Anthranilate 1, 2 dioxygenase (β subunit). Dendogram is sketched by MEGA 4.0 software.
6.3.6 Expression of recombinant Anthranilate 1, 2 dioxygenase β (rANT β)

Ni-NTA based column used for purification of rANT β purchased from Bangalore Genei. In single purification, expression was not clear, but when the eluted product was re-eluted from column the expression of rANT β appeared, which was confirmed by MALDI-TOF.

![Image of Ni-NTA Column based purification of rANT β cloned in pQE30 in E. coli BL21 after 6 hours of induction. In lane 1, crude lysate; lane 2, single purification; and in lane 3, repeated purification from the Ni-NTA column was loaded. White arrow indicated the rANT β.]

Figure 7.11: Ni-NTA Column based purification of rANT β cloned in pQE30 in E. coli BL21 after 6 hours of induction. In lane 1, crude lysate; lane 2, single purification; and in lane 3, repeated purification from the Ni-NTA column was loaded. White arrow indicated the rANT β.

7.4 DISCUSSION

Catabolic genes involved in the metabolism of Dibenzofuran have been already reported in many strains such as Sphingomonas HH69 (Harms et al., 1995), Sphingomonas sp. RW1 (Wittich, et al., 1992), Pseudomonas sp. strain CA10 (Sato et al., 1997), Terrabacter sp. (Omori et al., 1997; Schmid et al., 1997), Pseudomonas aeruginosa and Xanthomonas maltophilia (Ishiguro et al., 2000).

In few strains, it is found that the catabolic genes were dispersed over the genome without any clustering like in Sphingomonas sp. RW1 (Wittich et al., 1992; Armengaud et al., 1998). It can be said that these catabolic genes may or may not be clustered. Catabolic genes are well clustered in Pseudomonas sp. strain CA10 (Sato et al., 1997). In the clustered genes, if a fairly small fragment
of DNA is cloned, whole cluster of genes can be extracted through various techniques for example shotgun cloning. In our study, fortunately, we found anthranilate enzyme which is an active member of previously reported dibenzofuran catabolizing enzymes. The ~250bp fragment, coding for it, has been cloned in pQE30, an expression vector.

Anthranilate dioxygenase-β subunit (ant β) enzyme is very useful for degradation of dibenzofuran because it converts salycilic acid to cathechol which leads to cis cis-muconic acid directly. The anthranilate dioxygenase has [2Fe-2S] domain which is a catalytic center for electron transfer in oxygen molecule to form active oxygen for degradation (Nojiri et al., 2001). The degenerate primers based on MALDI and MS/MS data were designed. Fortunately the idea got clicked; ant β was amplified which is further confirmed after sequencing with degenerate primers. The sequence was also closely related with benzoate dioxygenase and ring hyroxylase.

The expression activities were checked and purified by single step purification by His-tagged anthranilate dioxyegnase with Ni-NTA column which was further confirmed by MALDI-TOF. The molecular weight in expression of ant β was found to be slight lesser than previous one. It is about ~19kDa. The ant β has been reported in strains but very few reports are available in terms of MALDI based cloning. This report, upto my knowledge, is the first report. The cloning of ant β will give several dimensions to future aspect of research. Firstly, the strain can be identified in mixed and complex population using MALDI. Secondly, based on this gene, FISH can also be performed in order to track our strain during in-situ bioremediation. Thirdly, based on this, whole cluster of catabolic genes can be traced out. The gene, ant β will work as probe provided, it should be well clustered like TOL plasmid. Its further amplification of the gene and their transcriptional regulation will enlighten the entire network that is coded for degradation.

There are only three system of the strain Sphingomonas sp. RW1 (Wittich et al., 1992), Pseudomonas sp. strain CA10 (Sato et al., 1997), Terrabacter sp.(Omori et al., 1997; Schmid et al., 1997) of which detailed study have
already been done. The research is very much required in this field for this strain, because unlikely to other strains, this strain has been isolated from site, polluted with dibenzofuran, and dominate during process of adaptation. This further research should be looked at that spectrum of its substrate and metabolites too. During proteome analysis, the transposon related proteins (Tn5 transposes) were found to be in high expression which is responsible for horizontal transfer and distribution of the genes throughout the community correlated with biofilm activity and biodegradation of dibenzofuran.

In conclusion, antβ was cloned and expressed successfully which will pave the way as molecular marker for getting whole catabolic cluster.