Cloning and sequencing of some of the gene(s) involved in $p$-nitrophenol degradation in *Ralstonia* sp. SJ98
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Introduction

Nitroaromatic compounds, including nitrophenols, are widely distributed in environment because of their extensive use in industry and in daily life and have become common pollutants soil and in water bodies. p-nitrophenol or 4-nitrophenol is an important member of the nitrophenols group. This chemical is a manufactured item that does not occur naturally in the environment (ATSDR, 1992). p-Nitrophenol has applications in the manufacture of pesticides, dyes/ pigments, engineering polymers and pharmaceuticals. It is used as fungicide for control of mold on leather and in organic synthesis (Montgomery and Welkon, 1990). p-Nitrophenol is also used in products used by the military (USEPA, 1997) and in the manufacture of acetaminophen, a non-aspirin pain reliever (Bhatti et al., 2002). It is also a major urinary metabolite of parathion and can be used as a biomarker of human exposure since it is a breakdown product of pesticides including parathion and fluoridifen (Rogers and Emon, 1993). Most nitrophenols, including p-nitrophenol, enter the environment during manufacturing and processing. It readily breaks down in surface waters but takes a long time in deep soil and in groundwater. Nitrophenols are toxic to plants, animals and humans due to their stability and result in bio-accumulation as they remain in the environment for long durations. Animal studies suggest that p-nitrophenol may cause a blood disorder (ATSDR, 1992) and acute exposure of p-nitrophenol may cause irritation of the skin, eyes, nose and throat, headache, loss of consciousness, drowsiness, nausea, cyanosis, liver and kidney damage, methemoglobinemia, central nervous system depression, dyspnea, sweating, muscular weakness, fatigue, abdominal cramps, dermatitis, corneal damage and hypothermia (http://bsd.eme.msu.edu/). According to the Standards for Chemical Products in UK, the use of p-nitrophenol is no longer recommended (UK MOD website) and it is one compound amongst the eleven phenolic compounds that have been listed by USEPA as priority pollutants (Keith and Telliard, 1979). Traditional wastewater treatment techniques include activated carbon adsorption, chemical oxidation, advanced oxidation systems, filtration, etc. However, in each technique there are limitations and disadvantages. For instance, activated carbon adsorption only
involves phase transfer of pollutants without decomposition but this induces another pollution problem (Juan, 1984). Chemical oxidation is unable to mineralize all organic substances and is only economically suitable for the removal of pollutants in high concentrations (Eisenhauer, 1968; Shibaeva et al., 1969; Moza et al., 1988).

In view of its toxic effects, efforts have been made to examine the removal of \( p \)-nitrophenol from effluents via biological means which is apparently a cleaner and cheaper process. Research has been carried out to investigate the \( p \)-nitrophenol degradation kinetics and its biochemical pathways using pure cultures (Nishio and Spain, 1993; Jain et al., 1994; Loser et al., 1998; Zhao and Ward, 1999; Bhushan et al., 2000) or mixed cultures (Wiggins and Alexander, 1988). However, only a little knowledge is available on the genetic and molecular regulation of \( p \)-nitrophenol degradation. In order to understand the molecular regulation of \( p \)-nitrophenol degradation, a \(~6.8\, \text{kb}\) fragment containing some of the gene(s) involved in \( p \)-nitrophenol degradation was cloned in pBluescript KS (II) and sequenced (GenBank Accession No. AY574278) from a \( p \)-nitrophenol degrading organism \( Ralstonia\) sp. SJ98 (Pandey, 2004). The cloned fragment encoded benzenetriol dioxygenase and maleylacetate reductase that are known to be involved in the lower degradation pathway. In the present study attempts were made to clone the other gene(s) encoding the upper pathway enzymes of \( p \)-nitrophenol degradation via the construction of a genomic DNA library of strain SJ98.

**Catabolic pathways for the degradation of nitrophenols**

Many pure cultures of bacteria have been isolated for their ability to utilize nitrophenol isomers as the sole source of carbon and energy for growth. The metabolically productive pathways include those involving either initial reduction of the nitro group prior to removal from the aromatic ring or direct removal as nitrite via oxygenolytic cleavage. The simplest pathway for nitrophenol degradation is perhaps that for \( o \)-nitrophenol involving a single enzyme for converting the substrate into a \( \beta \)-ketoadipate pathway intermediate (Fig. 1). The initial oxygenase was purified and shown to require 2 mol of NADPH as cofactor for the reaction (Zeyer and Kearney, 1984; Zeyer and Kocher, 1988). This suggests that the enzyme initially removes the nitro group from \( o \)-nitrophenol to form \( o \)-benzoquinone with a further reduction to form catechol. Two metabolic pathways have been described for \( m \)-nitrophenol degradation, wherein, the initial enzymatic pathway is NADPH-dependent partial reduction of the nitro group to form hydroxyxalamino moiety. In \( P.\ putida\) B2, \( m \)-hydroxylaminophenol is
converted to 1,2,4-trihydroxybenzene (1,2,4-benzenetriol, hydroxyquinol) via a lyase with the release of ammonia (Meulenberg et al., 1996). *Ralstonia eutropha* JMP134, on the other hand, converts the *m*-hydroxylaminophenol to aminohydroquinone through the action of a mutase (Schenzle et al., 1999) (Fig. 1).

![Catabolic pathways for the biodegradation of *o*-nitrophenol and *m*-nitrophenol.](image)

*A few bacterial strains have been isolated for their ability to degrade *p*-nitrophenol. Simpson and Evans (1953) first reported the preliminary evidence of oxidative degradation of *p*-nitrophenol using a *Pseudomonas* strain isolated from biological waste treatment. Initial work by Spain and Gibson (1991) with a *Moraxella* sp. species elucidated the entire metabolic pathway. *p*-Nitrophenol is degraded by an initial monooxygenase attack with the concomitant release of nitrite and production of hydroquinone. The reaction in crude cell extracts required 2 mol NADPH for the oxidation of 1 mol of *p*-nitrophenol. The benzoquinone is then reduced to hydroquinone by a quinone reductase at the expense of additional NADPH. Subsequently hydroquinone was degraded by the *Moraxella* sp. via ring fission to form γ-hydroxymuconic semialdehyde (Spain and Gibson, 1991; Leung et al., 2005). The ring-
cleavage product is then converted to maleylacetate by a dehydrogenase; maleylacetate is further reduced to β-ketoadipate and TCA cycle intermediates. Hanne et al. (1993) showed that *Arthrobacter aurescens* TW17 degraded *p*-nitrophenol via hydroquinone following exposure to *p*-nitrophenol, although it converted *p*-nitrophenol to 4-nitrocatechol upon induction with phenol, *m*-nitrophenol or *p*-cresol. A catabolic pathway for *p*-nitrophenol involving initial ring hydroxylation has been proposed for *Arthrobacter* sp (Jain et al., 1994). In this case *p*-nitrophenol is first hydroxylated to 4-nitrocatechol by an initial hydroxylase (Fig. 2). In a second monooxygenase catalyzed step, 1,2,4-benzenetriol is formed with the concomitant release of nitrite. The benzenetriol is further cleaved into maleylacetate via 1,2,4-benzenetriol dioxygenase; maleylacetate is further metabolized via β-ketoadipate into TCA cycle intermediates. Chauhan et al. (2000) elucidated a novel *p*-nitrophenol degradation pathway in *A. protophormiae* RKJ100 and consequently it has been included in the University of Minnesota Biocatalysis/Biodegradation Database.

![Figure 2](image_url)  
*Figure 2.* Catabolic pathway(s) for the degradation of *p*-nitrophenol. (1) *Moraxella* sp. degrades *p*-nitrophenol through hydroquinone and β-ketoadipate. (2) *Arthrobacter* sp. JS443 degrades *p*-nitrophenol through 1,2,4-benzenetriol and β-ketoadipate.

**Molecular basis for *p*-nitrophenol degradation**

Very little work has been carried out on elucidating the molecular basis of *p*-nitrophenol degradation. Initial studies have suggested that genes encoding *p*-nitrophenol degradation are located on plasmids in case of *A. aurescens* TW17 (Hanne et al., 1993) and *B. cepacia* RKJ200 (Prakash et al., 1996). Bang and Zylstra (1997)
cloned a ~19 kb NotI fragment containing some of the gene(s) involved in p-nitrophenol degradation. In association with the hydroquinone pathway, the p-nitrophenol degradation genes were cloned from *Pseudomonas* sp. strain ENV2030 and *Pseudomonas putida* JS444 (Zylstra et al., 2000). In the case of the hydroxyquinol pathway catabolic gene(s) for degradation of p-nitrophenol have not been reported. The only nucleotide sequence of in p-nitrophenol degradation genes (*nphA1, nphA2*) thus far reported is from *Rhodococcus* sp. strain PN1. The gene products NphA1 and NphA2 are reported to convert p-nitrophenol to 4-nitrocatechol, but the genes involved in the further degradation of 4-nitrocatechol and its metabolites from strain PN1 have not been reported to date (Takeo et al., 2003). Therefore, more information about the catabolic genes involved in p-nitrophenol metabolism is still needed, and in particular, information about those genes that act on the hydroxyquinol pathway is considered to be highly desirable. Recently, Kitagawa et al. (2004) cloned three gene(s), namely *npcA, npcB, npcC* encoding some of enzyme(s) involved in p-nitrophenol degradation from *Rhodococcus opacus* SAO101. This gram-positive 4-NP degrader was originally isolated as a bacterium able to degrade dibenzo *p*-dioxin (Kimura and Urushigawa, 2001). The strain also degrades biphenyl, naphthalene, phenol, benzene, dibenzofuran, dibenzo-*p*-dioxin, and 4-NP. However, the molecular regulation of gene(s) involved in p-nitrophenol degradation has not yet been completely elucidated and the enzymes involved have not been purified and characterized. Therefore, this area of research still holds considerable challenge.

### Materials and Methods

#### Bacterial strains, plasmids and culture conditions

*Ralstonia* sp. SJ98 was grown in nutrient broth or in mineral salt medium with 1% succinate (w/v) at 30°C. This strain was chosen because a ~6.8 kb bp fragment of genomic DNA was cloned from this organism (Pandey, 2004) that encoded two gene(s) of the lower degradation pathway of p-nitrophenol. *E. coli* strains were grown in LB at 37°C unless specified otherwise. Ampicillin was added to a final concentration of 100 µg/ml. For functional screening of the recombinant cosmid clones in *E. coli*, the clones that showed positive signals in the hybridization experiments were plated on to selective
plates containing 0.2 mM \( p \)-nitrophenol or 4-nitrocatechol, 10 mM sodium succinate and 5 mM thiamine. The plates were incubated in 30°C.

**Drop assay**

The recombinant cosmid clones were screened for their chemotactic ability using drop assay which has been standardized in the laboratory (Samanta et al., 2000; Pandey, 2004). Drop assay was performed using the cosmid clones of strain SJ98 that showed the presence of gene(s) involved in the degradation of \( p \)-nitrophenol following hybridization studies. The strain SJ98 was used as positive control and \emph{E. coli} DH5\(\alpha\) was used as negative control for this assay. Chemotaxis was checked against \( p \)-nitrophenol and 4-nitrocatechol using drop assay using the protocol mentioned above.

**Resting cell studies**

Resting cell studies were performed using a \( p \)-nitrophenol induced culture of one of the test clones of the SJ98-cosmid library; strain SJ98 was taken as positive control and \emph{E. coli} DH5\(\alpha\) was taken as negative control. The study was conducted according to protocols standardized in the laboratory (Pandey, 2004). Samples were withdrawn at various time intervals and analyzed using high performance liquid chromatography (HPLC) equipped with a RP-8 column (Waters) and with a PDA detector (Pandey, 2004.)

**Isolation of DNA (genomic, plasmid and cosmid)**

Genomic DNA was isolated from strain SJ98 using a genomic DNA isolation kit (QIAGEN, Germany). Plasmids were isolated from \emph{E. coli} clones using standard protocols of alkali lysis (Sambrook et al., 1989). For sequencing purposes since purified DNA was required, plasmids were isolated using Miniprep kit (QIAGEN, Germany). Cosmid DNA was isolated from \emph{E. coli} clones of the genomic DNA library using the cosmid DNA isolation kit (DNA Technologies, Inc., USA) according to manufacturer's instructions. Kits were used for isolation of DNA whenever purified DNA was required for the subsequent steps.

**Polymerase chain reaction (PCR) and DNA sequencing**

For PCR, template DNA concentration was \( \sim 50 \) -100 ng in case of genomic DNA and \( \sim 25 \) ng in case of \emph{E. coli} clones. In order to amplify the cloned benzenetriol dioxygenase gene and aldehyde dehydrogenase gene from strain SJ98, the annealing
temperatures were set to 50 °C for the former and 60 °C for the latter enzymes. Deep Vent Polymerase was used for amplification of these genes. A 50 µl PCR reaction contained 1x ThermoPol Buffer, 0.2mM of each species dNTPs, 1 unit of DeepVent DNA Polymerase (New England Biolabs, MA), template DNA and 40 pmol of each primer. The standard temperature program consisted of the following steps: initial denaturing at 95°C for 5 minutes followed by 30 cycles of: 95°C for 30 sec (denaturing), 50°C or 60°C (annealing) for 1 minute (depending on gene) and 75°C (extension) for 1 minute, followed by an final extension step of 75°C for 5 minutes. Primers used have been listed in Table 1; all were procured from Biobasics (Canada) and have been used for amplification (100ng/50 µl reaction mix) and also in sequencing reactions (10 ng/10 µl reaction mix). For colony PCR, freshly inoculated bacterial colonies were taken with a toothpick and lysed in 100 µl of distilled water. 1 µl of the lysate was added to the PCR reaction mix.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Gene amplified</th>
<th>Primer sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>HQ_F</td>
<td>540 bp 1,2,4-Benzene triol</td>
<td>5'-AGG AGT TCA TCC TGC T(G/C)(A/T) G-3'</td>
</tr>
<tr>
<td>HQ_R</td>
<td>dioxynogenase (partial)</td>
<td>5'-CGC AC(GC) CCG AAC AC(A/T) GCG TC-3'</td>
</tr>
<tr>
<td>ADH_F</td>
<td>1.2 kb Aldehyde dehydrogenase</td>
<td>5'-ATGCAAACGCAACTCTTTACGCAG-3'</td>
</tr>
<tr>
<td>ADH_R</td>
<td></td>
<td>5'-CTTTCAACGTGGATAGTAAGGGGGAAT-3'</td>
</tr>
<tr>
<td>BtD_F</td>
<td>0.8 kb 1,2,4-Benzene triol</td>
<td>5'-ATGAACGAACACCTCACGCAA TC-3'</td>
</tr>
<tr>
<td>BtD_R</td>
<td>dioxygenase</td>
<td>5'-AGCTTTTCACGCAGCCTTCACGAG-3'</td>
</tr>
<tr>
<td>M13F</td>
<td>Vector-specific primers for</td>
<td>5'-GTGTTCGCCAGTCACGAC-3'</td>
</tr>
<tr>
<td></td>
<td>sequencing for pUC18</td>
<td>5'-CAGGAAACAGCTATGAC-3'</td>
</tr>
<tr>
<td>M13R</td>
<td>Vector-specific primers for</td>
<td>5'-AATTAACCCTCACTAAAGG-3'</td>
</tr>
<tr>
<td></td>
<td>sequencing for SuperCos I</td>
<td>5'-GTAATACGACTCAGTAGGGC-3'</td>
</tr>
</tbody>
</table>

Table 1. Sequences of oligonucleotide primers used to amplify partial 1,2,4-benzenetriol 1,2-dioxygenase, aldehyde dehydrogenase and vector specific primers for SuperCos I and pUC18 (+).

Sequencing was performed with vector specific primers using an automated DNA sequencer (ABI PRISM, model 377, version 3; Applied Biosystems). Vector-specific primers were used because gene(s) cloned in plasmids or cosmids were to be sequenced. After sequencing, database searches were performed using the BLASTX service of the
National Center for Biotechnology Information (NCBI) to determine the homologous gene(s) and to speculate their putative function.

**Southern hybridization, colony blot hybridization and slot blot hybridization**

Southern hybridization was performed using the protocol standardized in the laboratory (Pandey, 2004). For DNA southern hybridization 8 µg genomic DNA of strain SJ98 was digested with suitable restriction enzyme(s) and subjected to gel electrophoresis. Separated DNA fragments were transferred from agarose gel to Hybond-N+ Nylon membrane (Pharmacia Amersham Biotech, USA). For DNA dot blot plasmid DNA (10 ng) was denatured by boiling for 5 min and chilled immediately on ice. After 5 min the DNA was blotted to the Hybond N+ nylon membrane. Following cross linking the blotted DNA to the Hybond-N+ membrane in UV for 5 min the membrane was probed with [α-32 P]-dATP (BARC, Mumbai, India) labeled DNA fragments. DNA probe was prepared by elution of the 540 bp PCR fragment of benzenetriol dioxygenase (Pandey, 2004) from agarose gels. Hybridization was performed at 42°C in Gold Hybridization Buffer (Pharmacia Amersham Biotech, USA). The membrane was then washed twice in a solution of 1X SSC (Sambrook et al., 1989) containing 0.1% sodium dodecyl sulphate (SDS) at 50°C (15 min each) and once in a solution of 0.1X SSC containing 0.1% SDS at room temperature (15 min). The membrane was then exposed to X-ray films (Hyperfilm, Pharmacia Amersham Biotech, USA). The results of the blots were visualized using Molecular Imager® FX system (BioRad, Hercules, CA) and the Quantity One Software (BioRad, Hercules, CA).

For colony hybridization, the recombinant colonies were picked and patched on nutrient agar plates containing ampicillin and incubated at 30°C overnight. The colonies were transferred onto a Hybond N+ membrane sized according to the petri-plate to be used for probing. The nylon membrane was then treated sequentially with Sol A (10% SDS), Sol B (0.5 N NaOH, 1.5 M NaCl), Sol C (0.5 M Tris-Cl, 1.5 M NaCl, pH 7.4) and Sol D (2X SSC) for 15 min each. The membrane was then UV-crosslinked, hybridized with the 540 bp fragment of benzenetriol dioxygenase gene or 1.4 kb aldehyde dehydrogenase gene (Pandey, 2004) and washed as mentioned above. These gene(s) were encoded by the ~6.8 kb bp fragment of genomic DNA of strain SJ98 as mentioned earlier. Results were analyzed as in the case of Southern blotting experiments as indicated above.
Plasmid DNA isolated from the white clones were blotted onto a Hybond N+ membrane using PR648 Slot blot filtration manifolds (Amersham Pharmacia Biotech. Inc., USA) and probed with both the probes mentioned above (Pandey, 2004). The membrane was then washed two times at room temperature in 2X SSC (diluted from 20X stock [3 M sodium chloride, 0.3 M Sodium citrate pH 7.0]) followed by two washes at 50°C in 0.1X SSC (Sambrook et al. 1989) and treated as in case of southern blotting experiment.

**Probe stripping and re-hybridization**

In order to screen the same batch of clones by two different probes, probe stripping and rehybridization was carried out. For probe stripping, the blot was dipped in stripping solution (0.4 M NaOH) for 45 min at 45°C and then into neutralization solution (0.1X SSC, 0.1% SDS, 0.2 M Tris.Cl (pH 7.5)) for 15-30 min. The blot is then treated with pre-hybridization solution for 1-2 h and the next probe was used.

**Construction of genomic DNA library**

Partially Sau3AI-digested genomic DNA of *Ralstonia* sp. SJ98 (DNA fragment size 3-15 kb) was ligated with BamHI-linearized vector pUC18 (MBI Fermentas, GmbH, Germany) at 16°C overnight. The vector map of pUC18/19 and the sequence of the multiple cloning site has been shown in figure 3. The ligation mixture was electroporated into electro-competent cells of *E. coli* DH5α (Sambrook et al., 1989). The transformants were selected on LB-ampicillin agar plates containing *isopropylthiol-β-D-galactoside* (IPTG) and *5-bromo-4-chloro-3-indoyl-β-D-galactoside* (X-Gal). The white colonies were thereafter picked and patched onto LB-ampicillin plates and also preserved in 10% glycerol vials in -70°C. The clones were checked for the size of inserts by digestion with *Hind*III restriction enzyme.

A genomic DNA library of strain SJ98 was also constructed in vector SuperCos I (Fig. 4) (Stratagene, CA) by DNA Technologies, USA. For this the restriction fragments of Sau3AI-digested genomic DNA of strain SJ98 (size fractionated and selected ~32-40 kb) was ligated to the *XbaI*-digested vector and packaged using Gigapack III gold packaging extract (Stratagene, CA). The packaged DNA was shipped to the laboratory. 1µl of the mixture was used to transform *E. coli* DH5α electro-competent cells (50 µl) plated on LB-ampicillin plates and the plates were incubated at 30°C.
Figure 3. Circular map and features of the pUC18/19 phagemid vector. The complete sequence and is available at GenBank (Accession no. VB0025) and the web-site of MBI Fermentas (http://www.fermentas.com/techinfo/nucleicacids/mappuc1819.htm).

Figure 4. Circular map and features of the SuperCos 1 cosmid vector. The complete sequence and list of restriction sites are available at www.stratagene.com.
Some colonies were picked and patched on LB-ampicillin plates and the rest were preserved as 10% glycerol stocks in -70°C. Few clones were randomly picked up and recombinant cosmid DNA was extracted and digested to check the presence of inserts. Screening of the clones was initially carried out via colony blot hybridization.

Chemicals

*p-Nitrophenol, 4-nitrocatechol and ampicillin were purchased from Sigma, USA. For hybridization experiments, the reagents, films and cassettes were procured from Amersham Pharmacia, USA. All other chemicals were of highest purity grade available locally.

Results and Discussion

Construction and screening of genomic DNA library in pUC18

The genomic DNA of strain SJ98 was digested with *Sau3AI* restriction enzyme and run on 0.8% agarose gel. Fragments of sizes ranging from ~3-15 kb were excised from the gel and purified using QIAQuick Gel Extraction column (QIAGEN, Germany) (Fig. 5A). This was then ligated into pUC18 vector (Fig. 5A) and electroporated into *E. coli* DH5α. The library of strain SJ98 in pUC18 vector thus constructed contained ~1500 recombinant clones. Some of the clones were digested with *BamHI* in order to check for the presence of inserts and to determine the size of inserts (Fig. 5B).

The recombinant clones were picked and patched on LB-ampicillin plates in duplicate. Clones from one of these plates were preserved in 10% glycerol at -70°C and the second plate was used for inoculating clones in LB (5 ml) vials (10 clones together) for isolation of plasmid DNA. In this way, plasmids were isolated from all the clones in fewer number of isolations and they were thereafter used for slot blot hybridization using two radiolabeled probes sequentially i.e. (i) partial benzenetriol dioxygenase gene, and (ii) aldehyde dehydrogenase gene amplified from the cloned ~6.8 kb genomic DNA fragment of strain SJ98 (Fig. 6). These gene(s) were amplified from the cloned ~6.8 kb fragment (pSJ262) using the gene-specific primers as shown earlier in Table 1. From the restriction map of this fragment it is evident that the aldehyde dehydrogenase gene(s) is located towards one end of the sequence whereas the benzenetriol dioxygenase is located further downstream from it (Fig. 6).
Figure 5. (A) Gel photograph showing the digested vector pUC18 (lane 1) and partially digested and gel-excised fragment of genomic DNA isolated from strain SJ98 (lane 2). Lane M shows molecular weight marker (λ DNA digested with HindIII). (B) Restriction digestion of some of the clones of the library. The clones were digested with BamHI (lane 1-7) and lane M represents 1 kb ladder.

Figure 6. Organization of the Open reading frames (ORFs) and restriction map of the ~6.8 kb cloned fragment from Ralstonia sp. SJ98 (pSJ262) involved in p-nitrophenol degradation (Pandey, 2004).

Therefore, both the probes were used such that the clones screened thereafter would contain some other gene(s) downstream and/or upstream of these gene(s). Also, earlier experiments had shown that benzenetriol dioxygenase gene has only one copy number in the genome (Pandey, 2004) and therefore it was assumed that any clone containing an insert of 7 kb or more, and hybridizing with both these probes would
encode some other gene(s) involved in the p-nitrophenol degradation pathway. After screening the clones with the benzenetriol dioxygenase gene (Fig. 7), the blot was stripped, washed and re-hybridized with the aldehyde dehydrogenase gene.

![Figure 7](image)

**Figure 7.** A representative blot during the preliminary phase of screening genomic library clones. The blots were hybridized with benzenetriol dioxygenase and then aldehyde dehydrogenase gene(s). A1 and A12 represent positive controls (genomic DNA of SJ98) and D12 represents negative control (genomic DNA of *E. coli* DH5α containing pUC18).

![Figure 8](image)

**Figure 8.** (A) One representative colony blot during the second phase of screening clones of the genomic DNA library. (B) Positive clones were also screened using slot blot hybridization as shown in the blot. The (+) sign indicates the position of genomic DNA of strain SJ98 used as positive control. Genomic DNA of *E. coli* DH5α (transformed with pUC18) was used as negative control (last slot, 11nd row). Arrows indicate positive signals.
The clones showing positive signals with both probes were selected and thereafter the individual clones were then picked up from the group of 10 clones (primary screening) as indicated above and patched on LB-ampicillin plates. These clones were screened using colony hybridization with positive and negative control as mentioned in figure 8A. The clones that showed hybridization signals were once again checked for slot blot hybridization (Fig. 8B) and colony PCR.

Figure 9. Final blot showing hybridization of two clones (A2, A4) with the aldehyde dehydrogenase probe. A1, A11 and A12 represents different concentrations of SJ98 genomic DNA. A3, A5-10 also represent clones that were suspected to be positive in colony blot hybridization; they did not show positive signal in slot blot hybridization.

Figure 10. Amplification of aldehyde dehydrogenase gene from the positive clones (lane 1, 2). Lane 3 shows amplification obtained by using pSJ262 as positive control. M represents 1 kb ladder.

Finally, after two rounds of screening via colony blot followed by slot blot hybridization and colony PCR, two clones were obtained that gave positive signals with the aldehyde dehydrogenase probe (Fig. 9). These clones (designated as clone 1-ADH and clone 2-ADH) also showed an amplification of the expected size of ~1.4 kb using ADH_F and ADH_R primers (Fig. 10). For positive control pSJ262 was used as
template. The clones were digested with HindIII to determine the approximate size of the insert (Fig. 11). Unfortunately there were no positive clones encoding benzenetriol dioxygenase, as shown by the results of the final slot blot hybridization and colony PCR in which we obtained non-specifically amplified products (data not shown).

![Figure 11](image)

**Figure 11.** Gel showing the restriction digestion of the positive clones obtained from the genomic DNA library. Lane 1 shows pUC18 digested with HindIII, lane 2 and lane 3 shows recombinant clones. M represents the 1 kb ladder.

Clone 1-ADH and clone 2-ADH showed an insert size of app. 5.5 kb and 2.5 kb respectively. The two clones were thereafter sequenced using vector-specific primers (M13F and M13R) from both ends. The sequences obtained (in 5'-3' orientation) are as follows:

**Clone 1-ADH (primer used: M13F)**

```
GGTCGACCTCGTCCCATCGCGACAAGCGAATCGCTGCAGCGGCTACGCGCGATTCGACGCTGACG
ATCGAATCGTTAACAAGCGAATCGTTATCGCGAGGCGATCGCGAGGCGATCGCGAGGCGATCGCG
ACGCGACGACGACGACGACGACGACGACGACGACGACGACGACGACGACGACGACGACGACGACG
ACGCGACGACGACGACGACGACGACGACGACGACGACGACGACGACGACGACGACGACGACGACG
ACGCGACGACGACGACGACGACGACGACGACGACGACGACGACGACGACGACGACGACGACGACG
ACGCGACGACGACGACGACGACGACGACGACGACGACGACGACGACGACGACGACGACGACGACG
ACGCGACGACGACGACGACGACGACGACGACGACGACGACGACGACGACGACGACGACGACGACG
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When the above sequences were compared with other existing sequences in the database using BLASTX tool (NCBI) it was found that clone 1-ADH showed homology with ATPase transporter gene but not with aldehyde dehydrogenase. However, ADH-2
showed homology with aldehyde dehydrogenase gene (sequence obtained with M13F) and therefore it could be sequenced further to obtain gene(s) located upstream to this gene.

**Screening of genomic DNA library constructed using SuperCos I vector**

A genomic library of strain SJ98 was also constructed in SuperCos 1 vector containing an average insert size of ~32-40 kb and was obtained from DNA Technologies (Gaithersburg, USA). The packaged DNA supplied by them was electroporated in the *E. coli* DH5α host cells and plated on LB-ampicillin plates. Three thousand recombinant clones were picked and patched on to LB-ampicillin plates and thereafter used for screening for the presence of *p*-nitrophenol degradation gene(s). The probe used was a 540 bp partial fragment of benzenetriol dioxygenase gene as mentioned above in colony and blot blot hybridization experiments. All the 3000 clones were screened using colony hybridization for which 50 colonies were blotted on a single membrane (preliminary screening) (Fig. 12A). The positive clones obtained on each blot were picked up and patched onto another plate (clone no. 957, 2012, 2825, 88, 946, 1956, 1264, 1276, 1881) and these clones were screened again by colony hybridization (secondary phase of screening) (Fig. 12B). Thereafter, the positive clones were selected for slot blot hybridization. For this purpose cosmid DNA was isolated from these clones using the cosmid DNA isolation kit and the DNA was blotted onto nitrocellulose membrane and hybridized with the same 540 bp fragment of benzenetriol dioxygenase (Fig. 13). The results of slot blot hybridization re-confirmed the results of colony blot hybridization (Fig. 13) and 9 clones were found to be showing strong hybridization signals with the probe. This DNA was also used for restriction digestion with *BamHI* in order to determine the approximate size of the recombinant cosmids and the restriction pattern of each of them (Fig. 14).

All the 9 clones were subjected to restriction digestion by *BamHI* and the restriction patterns obtained were compared visually. The size of the vector SuperCos 1 (7.9 kb) was subtracted from the fragment size(s) of the clones to obtain the approximate size of the inserts as listed in Table 2. The actual number of the clones i.e. the number out of initial 3000 clones have also been recorded in Table 2. The digestion patterns (Fig. 14) showed that out of 9, there were 6 different types of clones and 4 of them had the same restriction pattern (Table 2). This suggested that at least 6 of the clones carried different inserts and encoded the gene(s) for benzenetriol dioxygenase.
Figure 12. Colony blots performed on clones of SJ98 cosmid DNA library. The blots were hybridized with the 540 bp partial gene of benzenetriol dioxygenase gene. Blot A represents preliminary round of screening and blot B represents secondary phase of screening where only the suspected positive colonies were used for blotting. (+) sign represents the position of positive control (genomic DNA of strain SJ98). Genomic DNA of *E. coli* DH5α was used as negative control (opposite to +ve control). Arrows represent hybridization signals with the probe.

Figure 13. Slot blot hybridization performed on the cosmid clones that were positive in the second round of colony hybridization. Slots A1 and H2 represents positive control (genomic DNA of strain SJ98). In lane 3 slots (A-C) contained DNA from clones that did not show hybridization with the benzenetriol dioxygenase probe (negative control).

Figure 14. Restriction pattern of cosmid clone no. 2012 (L1), 2825 (L2), 946 (L3), 1956 (L4), 1276 (L5) and 1264 (L6). Lane M shows molecular weight marker (λ DNA digested with *HindIII*) and molecular weight of individual bands have been indicated alongside (in kb).
Table 2. Table representing the size of restriction fragments of the 9 positive clones and the approximate size of individual clones. Clones 957, 88, 946 and 1881 appeared to be identical based on the restriction patterns. Clone no. indicates the actual numbers of the colonies in the library.

<table>
<thead>
<tr>
<th>Clone no.</th>
<th>App. fragment sizes with BamHI (kb)</th>
<th>Total size (kb)</th>
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<tbody>
<tr>
<td>957</td>
<td>10, 9, 6, 4, 3, 3.5, 2</td>
<td>36</td>
</tr>
<tr>
<td>2012</td>
<td>10, 8.4</td>
<td>22</td>
</tr>
<tr>
<td>2825</td>
<td>11, 8, 3, 3.5</td>
<td>26</td>
</tr>
<tr>
<td>88</td>
<td>10, 9, 6, 4, 3, 3.5, 2</td>
<td>36</td>
</tr>
<tr>
<td>946</td>
<td>10, 9, 6, 4, 3, 3.5, 2</td>
<td>36</td>
</tr>
<tr>
<td>1956</td>
<td>10, 9, 7, 4, 3, 3.5, 2</td>
<td>37</td>
</tr>
<tr>
<td>1264</td>
<td>9, 8, 5, 4, 3.5</td>
<td>30</td>
</tr>
<tr>
<td>1276</td>
<td>10, 5</td>
<td>15</td>
</tr>
<tr>
<td>1881</td>
<td>10, 9, 6, 4, 3, 3.5, 2</td>
<td>36</td>
</tr>
</tbody>
</table>

Figure 15. PCR amplification of partial (540 bp) and complete benzenetriol dioxygenase gene (818 bp) from one of the positive clones (lane 1, 2). Amplification of aldehyde dehydrogenase gene (1.4 kb) from the same clone (lane 3). Lane 4, 5, 6 shows amplification of the same gene(s) under same conditions from the positive control (pSJ262). Lane M represents 1 kb DNA ladder.

In addition, to reconfirm the results of hybridization, partial and complete benzenetriol dioxygenase and aldehyde dehydrogenase gene(s) were amplified using primers HQ_F, HQ_R; BtD_F, BtD_R and ADH_F, ADH_R (Table 1). These results have been shown in figure 15. The complete benzenetriol dioxygenase gene was also sequenced so as to rule out the probability of non-specific amplification. Two of the positive clones having larger inserts were sequenced using vector-specific primers (T3
and T7) in order to elucidate the gene(s) located at either end of the insert. The sequences were aligned with the sequence of the ~6.8 kb fragment of strain SJ98 genomic DNA using Clustal X (Thompson et al., 1997) so as to determine the homology between these sequences. The sequences (5'-3' orientation) are as follows:

**Clone 88 T7 primer**

GTGTCACTGGGATCCTGATACCCATTCCATGCGCAGCGATCGA
GAAACCTTGTATAAAGAGGGCAGCCCGAGCGAAAGCGTCTCCATGTGATATCCGCCCGCGAA
GCCGCGCTTCGGATCGAACACGGATTCATCCTCGATGATGCCGGCCATCGTCGTGCCCTTGAA
CATCTCGACCTTGTCGTTGAACACGCCGTAGACCGAGCCTGTCGTGTGCCGCATGTAGTTGGC
GCCGACCTGGCCCGAGCCGTTCGCGAGGCCATCGGGGAACTTGCTCGACTGCGAGTTGAGCA
GGAGCCGCGCCGCTTCGATCGCATTGCCCGCCACCGAGCGACGCGCCCGCCTCTGCCGTTGC
AGCTTGCCGTCTCTGTGTAATAGACGAGACGGGCTCTGCGCTCGCTGTCGTACGTCATC
TTCGAGACATGCGATCGGCGGATCGTTGTGCCCTTGAGCTAGCCATGC

**Clone 88 T3 primer**

ACCACTCCAGGACCCCTTTTTAAGAGAGGTAGAGCGGATTCTTGCTGCCGGTTCGCCTTAAGA
CCATTCAAGGCTATCGATCTGGGCAGTCTTGCGCGAGGGTCTCAAGCCATTGCGCTCGCAGT
ACGACTACATCTGTAGTACTAGTACCGTCGCTGTCGTATATGAATTTGAACGCGCTGCTCGC
CCGCCGACGCGCTTCGGAATCTCGACTTCATTAGTTCGCTCGCGTTCTGGCGACTCTTCTCGGACGTTGCCGAAGACTTCCTTCCTTACGAGGAGGATAAGGTCTACG
ACTTCATCTCITATTTTTGCGAGAATGGTGATTAGCTACGCGCGCAATCAGTGGCCGCG
GGCAATGGGCGGGAAAGCCGATACCTCGGCATCGGGATCGCTCAGCTATCGCCGAG

**Clone 957 T7 primer**

TTTTTCTGCTGGGCCTGGCCACTGCTATCGCGCGGATGTGGTCCGGGCTGCTCGATCGCA
GGCGGCGGCTGCTGGCCTTCTTGTCGTCCTCGAGTTGCTTCGCCTTGATCGAGCC
CCCATCTTCCGGGTCCGCTTCGCCGATGCGGTAATTTCCCTTCCAGGGCCTTGAACAGATAGC
CGCGCGGGCCTGACCGTCACCTTGCCTTTCGTTGAGCTTGTATCGCGTGTATTCGATCGCCTGT
TGAGCCGCTCGTTGGTCCACACGTCGCGCCCTCCTGCGAGGCGCTCGAAGTCGGGGGTATC
GAAAGCGAATTCGTTGTGCAAGAAGCAGATACATCGGAATCCTGAATGCCGGCGCGCGCCCGG
GTTCTGCGCCCTTGCTCCTTCGTGAAGGCGGAAAACGGAATTTTCAGCCTTGGTCGGCCGGAT
CCGCGGCTCCCGGAGCCGCAGCGCGGCATCGCCGAGCGCGCTTTCGCGACGCGCGAGCGCGCG

32
Clone 95 7 T3 primer

TTTGGGGTATTTTAAGTGAAGACGGGCCAGTCGAGGTATTTTTTTTTCTTGTATTTGAATATTTTTTGC
CAGAGCGAGCGAGCCGTCGTCGTTCTTGATTCGCGCACCTTTTTTTAAAGGTGAGAGACCGAGGCCGAT
CAGACGCTCGTGCTCCGCCTTCGCAAAAGGCACGCTCACCGCGACGTGGCCGTAGCCGTCGCCATGTGTG
TATGGCTCGGCGCGTCCCTTGTTCCAGGTGAGTTCGAGCTCGGCGTTGGTTTCGTCGTTGCGCAGATAGG
CGAGCGTGAAGTCGGGAAAGTCCAGCCGGTGCGCGACGCCGAGATCGAAAGCCTTCTCGTAGAAATCGA
GCGAGCGTTGCAGGTCCTGCACGCGAATCATCGTGTGAATGAGCTTTGACATGAGAGCCTCCCCTTTTT
AGTGAAGTTTAAAGACAAGCGCGCGCGAATCGGTTAAATCGGGCGTACGGCTGCGCGCTCATCGCCCG
TCGAACGTTCGAATTCGATCATCGCGATAGTCGAACAGAAAGGGCACGGCGCGCCGCTCATCGCCCG
TCCGATGCCGAACGTTCGAATTCGATCATCGCGATAGTCGAACAGAAAGGGCACGGCGCGCCG

The sequences did not match with the previously cloned ~6.8 kb fragment (Pandey, 2004) suggesting that the ORFs encoded on the 6.8 kb fragment (pSJ262) were not located adjacent to the cloning site of SuperCosI (Fig. 16). This indicates the possibility that the insert cloned in the cosmid encoded some of the gene(s) of the upper \textit{p}-nitrophenol degradation pathway. Also, as gene(s) encoding degradative enzymes are often clustered (Spain et al., 2000; Takeo et al., 2003; Kitagawa et al., 2004), it was expected that the upper pathway gene(s) would be located upstream or downstream of the known ~6.8 kb fragment. Thereafter, functional screening of clones was carried out to determine the presence of the entire gene cluster in the cosmid clone.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure16.png}
\caption{Representation of one of the cosmid clones of strain SJ98 that contains gene(s) of the lower pathway for \textit{p}-nitrophenol degradation. The upstream (Seq1) and downstream (Seq2) of the ~6.8 kb known sequence is expected to encode the gene(s) of the upper pathway for \textit{p}-nitrophenol degradation. Sequences (1 kb) obtained with vector-specific primers (T3 and T7) did not match the previously cloned and sequenced ~6.8 kb fragment suggesting that this sequence is encoded away from the cloning site of SuperCosI (\textit{BamHI}).}
\end{figure}
Chapter 2

Functional screening of clones of cosmid library

Strain SJ98 has been reported to be capable of growing on various nitroaromatic compounds that are used as sole sources of carbon and energy by this organism. Additionally, it is also known to be chemotactic toward some of the nitroaromatic compounds (Samanta et al., 2000; Pandey et al., 2002). Bacterial chemotaxis is the movement of bacteria toward or away from a chemical gradient and some bacteria including strain SJ98 has been reported to be chemotactic toward various pollutants (Samanta et al., 2000; Pandey et al., 2002). Also, it is known from literature that gene(s) involved in degradation of pollutants have been reported to exist in cluster(s) in some organisms (Spain et al., 2000; Takeo et al., 2003; Kitagawa et al., 2004). Therefore, it was speculated that the cosmid clones that showed the presence of benzenetriol dioxygenase and aldehyde dehydrogenase, may encode gene(s) for the degradation of p-nitrophenol as well as some other nitroaromatic compounds and also receptor(s) involved in chemotaxis toward nitroaromatics. The clones were also checked for their chemotactic property using drop assay. However, the gene(s) responsible for this degradation could not be efficiently expressed in E. coli and so there were no visible changes in the plates containing nitroaromatic compounds, indicating the absence of transformed products. In drop assay, ring formation was not observed indicating that the colonies were not chemotactic toward p-nitrophenol and 4-nitrocatechol.

In order to detect the presence of the complete gene cluster for p-nitrophenol degradation in the clones, resting cell studies were performed with one of the clones of the library and the utilization of p-nitrophenol (0.3 mM) or 4-nitrocatechol (0.2 mM) was studied. The large number of cells used in resting cell studies result in cumulative expression of the degradative enzymes, thereby circumventing the problem of inadequate or low levels of gene expression in E. coli cells. HPLC analyses of samples collected at time intervals (zero h, 12 h, 24 h and 30 h) showed a significant change in the concentration of the nitroaromatic compound added. Figure 17 shows the depletion of p-nitrophenol with time by clone no. 88 and also shows the amount of p-nitrophenol remaining in the medium at each time point. SJ98 cells were used as positive control and strain DH5α was used as the negative control. p-Nitrophenol was degraded in 25 min by strain SJ98, whereas, DH5α could not deplete it even after 2 days. This experiment indicated that degradative enzymes were being expressed at low levels by the clones and that clone no. 88 encoded the entire p-nitrophenol degradation pathway. The results were
similar for 4-nitrocatechol degradation by resting cells of clone 88, however, owing to
greater toxicity of 4-nitrocatechol, the rate of degradation was lower even at 0.2 mM
concentration.

![HPLC results showing the depletion of p-nitrophenol by the cosmid clone 88 as
determined in resting cell studies. The concentration of p-nitrophenol was determined in these
samples at various time intervals and the chromatograms obtained have been overlayed for easy
comparison.](image)

<table>
<thead>
<tr>
<th>Component Summary Table</th>
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<tr>
<td><strong>SampleName</strong></td>
</tr>
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</tr>
<tr>
<td>2</td>
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<tr>
<td>3</td>
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<td>4</td>
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</tbody>
</table>

**Figure 17.** HPLC results showing the depletion of p-nitrophenol by the cosmid clone 88 as
determined in resting cell studies. The concentration of p-nitrophenol was determined in these
samples at various time intervals and the chromatograms obtained have been overlayed for easy
comparison.

There are two recent reports on cloning of gene(s) encoding the enzymes involved in
the degradation of p-nitrophenol and 4-nitrocatechol from Gram-positive organisms such as
*Rhodococcus opacus* SAO101 (Kitagawa et al., 2004) and *Rhodococcus* sp. PN1
(Takeo et al., 2003). The authors have been able to clone the entire gene cluster in the
former case and the first gene involved in the conversion of p-nitrophenol to
4-nitrocatechol in the latter case. However, this is the first instance for cloning of gene(s)
involved in degradation of p-nitrophenol and 4-nitrocatechol from a Gram-negative
organism i.e. strain SJ98.
References


32. UK MOD. Defence standardization website. www.dstan.mod.uk/dsmain.htm


