CHAPTER THREE
MATERIALS AND METHODS

3.1 BACTERIAL STRAINS

Various bacterial strains and plasmids used in the present study have been listed in Tables 3 and 4. Their relevant phenotypic properties, mode of derivation and source of procurement are also shown in these tables.

3.2 COMPOSITION OF MEDIA

The composition of the minimal medium (MM) used for growth of various organisms on different compounds as the sole source of carbon and energy is as follows:

- Na$_2$HPO$_4$ 2.0g
- KH$_2$PO$_4$ 1.0g
- (NH$_4$)$_2$SO$_4$ 0.4g
- MgSO$_4$.7H$_2$O 0.4g
- Trace elements solution 2.0ml
- distilled H$_2$O (dH$_2$O) 1000ml

In order to prepare the plates, agarose was added to a final concentration of 1.5%.

Composition of Trace Elements Solution (Kaul, 1992)

- Al(OH)$_3$ 0.10g
- SnCl$_2$.2H$_2$O 0.05g
- KI 0.05g
- LiCl 0.05g
- MnSO$_4$.4H$_2$O 0.08g
- H$_3$BO$_3$ 0.50g
- ZnSO$_4$.7H$_2$O 0.10g
- CoCl$_2$.6H$_2$O 0.10g
- NiSO$_4$.6H$_2$O 0.10g
**Table 3:**

**Bacterial strains used.**

<table>
<thead>
<tr>
<th>STRAIN</th>
<th>RELEVANT CHARACTERISTICS&lt;sup&gt;(a)&lt;/sup&gt;</th>
<th>PLASMID</th>
<th>PARENT STRAIN(S)</th>
<th>SOURCE/DERIVATION/REFERENCE</th>
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<td>Nap&lt;sup&gt;+&lt;/sup&gt; Sal&lt;sup&gt;+&lt;/sup&gt; Phen&lt;sup&gt;+&lt;/sup&gt; OPA&lt;sup&gt;+&lt;/sup&gt;</td>
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<td>This study</td>
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<td>PpG7</td>
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<td>NAH7</td>
<td>--</td>
<td>I.C. Gunsalus, University of Illinois, Illinois; Franklin et al. (1981)</td>
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<tr>
<td>KT2442</td>
<td>Nap&lt;sup&gt;+&lt;/sup&gt; Sal&lt;sup&gt;+&lt;/sup&gt; Phen&lt;sup&gt;+&lt;/sup&gt; OPA&lt;sup&gt;+&lt;/sup&gt; Rif&lt;sup&gt;r&lt;/sup&gt; m&lt;sup&gt;+&lt;/sup&gt;</td>
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<td>PaW1</td>
<td>Microbial Type Culture Collection, (MTCC), IMTECH, Chandigarh; Pickup et al. (1983)</td>
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<td>PaW340</td>
<td>Nap&lt;sup&gt;+&lt;/sup&gt; Sal&lt;sup&gt;+&lt;/sup&gt; Phen&lt;sup&gt;+&lt;/sup&gt; OPA&lt;sup&gt;+&lt;/sup&gt; Trp&lt;sup&gt;r&lt;/sup&gt; Str&lt;sup&gt;r&lt;/sup&gt; m&lt;sup&gt;+&lt;/sup&gt;</td>
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<td>This study; Treatment with mitomycin C</td>
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<td>RKJ1</td>
<td>This study; Treatment with NTG&lt;sup&gt;(b)&lt;/sup&gt;</td>
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<td>STRAIN</td>
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<td>PARENT STRAIN(S)</td>
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<td><em>Moraxella sp.</em></td>
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<td>S-17.1</td>
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<td>SK1592</td>
<td>Km' Amp'</td>
<td>pRK 2013</td>
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<td>C600</td>
<td>Km', 15kb EcoR1 fragment, encoding genes for the conversion of naphthalene to salicylate, cloned from naphthalene encoding plasmid pDTG1 and inserted into vector pKT230</td>
<td>pDTG 113</td>
<td>C600</td>
<td>D.T. Gibson, University of Iowa, Iowa; Serdar and Gibson (1989a, b)</td>
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Table 3: (Continued)

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<th>PARENT STRAIN(S)</th>
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<td>Cm&lt;sup&gt;+&lt;/sup&gt;, 12.05kb EcoRI-BglII fragment, carrying genes for the conversion of naphthalene to salicylate, from naphthalene-encoding plasmid NAH7 cloned into vector pMMB277</td>
<td>pRE731</td>
<td>S-17.1</td>
<td>Richard Eaton, Environmental Protection Agency, Florida; Eaton (1994)</td>
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(a) Nap, naphthalene; OPA, o-phthalate; Phen, phenanthrene and Sal, salicylate. The ability of the organisms to grow or not to grow on these carbon sources is denoted by superscript (+) or (-), respectively.

Pro<sup>-</sup> Thi<sup>+</sup> Trp<sup>-</sup> represent auxotrophy for the amino acids proline, thiamine and tryptophan, respectively.

Amp<sup>+</sup>, Cm<sup>+</sup>, Km<sup>-</sup>, Rif<sup>+</sup>, Str<sup>-</sup>, Tmp<sup>-</sup> and Tet<sup>-</sup> indicate the resistance to ampicillin (100μg/ml), chloramphenicol (30μg/ml), kanamycin (25μg/ml), rifampicin (150μg/ml), streptomycin (150μg/ml), trimethoprim (25μg/ml) and tetracycline (15μg/ml), respectively.

RecA<sup>-</sup>; homologous recombination abolished.

Restriction minus or restriction positive is denoted by r<sup>-</sup> or r<sup>+</sup>, respectively.

Modification minus or modification positive is denoted by m<sup>-</sup> or m<sup>+</sup>, respectively.
lac Z: fl-galactosidase activity abolished or present.
Chr: RP4-2: RP4-2 integrated into host chromosome.
(b) NTG: N-methyl-N'-nitro-N-nitrosoguanidine.
(c) First strain indicated is the donor in conjugation.

Table 3: (Continued)
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Table 4: Vectors used.
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<th>Markers/Characteristics***</th>
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<td>Erich Lanka, Max-Planck Institut fuer Molekulare Genetik, Berlin</td>
<td>pMMB22</td>
<td>8.8</td>
<td>Amp', Strr, Amp', and Tetr indicate the resistance to ampicillin (100 μg/ml), kanamycin (25 μg/ml), streptomycin (150 μg/ml), and tetracycline (15 μg/ml), respectively.</td>
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<td>Strr, Amp'</td>
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(a) Amp', Kmr, Strr, and Tetr indicate the resistance to ampicillin (100 μg/ml), kanamycin (25 μg/ml), streptomycin (150 μg/ml), and tetracycline (15 μg/ml), respectively.
Since naphthalene is volatile, it was provided in vapour phase in a closed container. The MM agar plates were inoculated with the test organism(s) and were kept in a closed container in which crystals of naphthalene had been placed (Barnsley, 1976b). Three different ways were employed in order to check growth on phenanthrene as the sole source of carbon and energy. They were: (1) The phenanthrene was added to the MM at a final concentration of 1mM before autoclaving and the plates were inoculated with the test organism(s) (Strandberg et al., 1986); (2) The phenanthrene was sprayed as 10% (w/v) solution in diethyl ether over the surface of MM agar plates previously inoculated with the test organism(s). The ether immediately vaporized from the agar surface at room temperature and a white, thin layer of phenanthrene remained on the surface of solid media (Kiyohara et al., 1982); (3) The cultures on NA plates were grown overnight before spraying phenanthrene as mentioned above. In the latter two cases, the ability of organism(s) to degrade phenanthrene was confirmed by the presence of clearing zones around the inoculated regions on the plates.

For growth of the organisms on various organic compounds including intermediates of naphthalene and phenanthrene degradative pathways, they were added to the MM agar plates at a concentration of 0.5-2.5mM depending on the compound; sodium succinate and pyruvate were added at a final concentration of 10mM. Stock solutions of all these compounds were sterilized separately and were added to the media just before use.

Nutrient broth (NB) was prepared by dissolving 13g of nutrient broth in 1000ml of dH₂O. The nutrient agar (NA) plates were made by adding 15g of purified agar as solidifying agent to 1000ml NB before autoclaving. Luria Bertani (LB) medium contained 10g tryptone, 5g yeast extract and 10g NaCl in 1000ml dH₂O. Purified agar was added to the medium at a final concentration of 1.5% before autoclaving for the preparation of LB agar plates.

The final pH of all the media used was adjusted to 7.4. The media were then
sterilized by autoclaving for 20 min at 121°C (15 pounds). Stock solutions of heat-labile amino acids and sugars were prepared by filtering through 0.22 μm Whatman filter membranes (Whatman Ltd, Maidstone, England). For growth and maintenance of auxotrophic strains, the media were supplemented with the required amino-acid(s) at a final concentration of 25 μg/ml. Whenever needed, the antibiotic(s) were added to the media at final concentrations as follows: kanamycin (Km) (25 μg/ml), streptomycin (Str) (100 μg/ml), ampicillin (Amp) (100 μg/ml), rifampicin (Rif) (100 μg/ml) and tetracycline (Tet) (15 μg/ml). In order to check the growth of organisms on heavy metal salts, the stock solutions were prepared in double distilled H₂O (ddH₂O) and were filter sterilized separately before adding to the media.

3.3 BUFFERS

The phosphate buffers used in enzyme assays were prepared by dissolving the required quantity of KH₂PO₄ in ddH₂O and the pH was adjusted with 10 M NaOH and then making up to the final volume with ddH₂O. For the preparation of Tris-HCl buffers, the required amount of tris(hydroxymethyl)methylamine (Tris base) was dissolved in ddH₂O, the pH was adjusted with 10 M HCl and the final volume was made up with ddH₂O. The molarity and pH of the phosphate and Tris-HCl buffers used are stated in the text.

For agarose gel electrophoresis, 50X Tris-acetate-EDTA (50X TAE) buffer was prepared by dissolving 242 g of Tris base, 100 ml of 0.5 M EDTA (pH 8.0) and 57.1 ml of glacial acetic acid in a final volume of 1000 ml ddH₂O (Maniatis et al., 1989). Tris-EDTA (TE) buffer consisted of 10 mM Tris-HCl and 1 mM EDTA (pH 8.0). For hybridization purposes, 20X sodium citrate-sodium chloride (20X SSC) buffer contained 3 M sodium chloride and 0.3 M tri-sodium citrate in ddH₂O (Maniatis et al., 1989). The composition of other buffers used has been described in the text.

3.4 ISOLATION AND CHARACTERIZATION OF WILD-TYPE STRAINS

Enrichment techniques were used to isolate various organisms capable of utilizing naphthalene or phenanthrene as the sole source of carbon and energy. One
gram of the soil sample(s), collected from known oil fields by scientists from IMTECH, Chandigarh, India, was resuspended in 100ml of MM. The naphthalene was provided in vapour phase and phenanthrene was added at a final concentration of 1mM. The samples were incubated at 30°C on an orbital shaker at 180 revolutions per minute (rpm) for one week. Ten ml of each sample was added to 90ml of fresh MM containing naphthalene or phenanthrene and the flasks were incubated at 30°C for another week. This subculturing was carried out for five cycles. Serial dilutions of the final cultures were then spread onto NA plates and incubated at 30°C. After overnight incubation, colonies with different morphology were picked up and patched onto MM agar plates containing naphthalene or phenanthrene, separately. The isolates growing on any of the above plates were further streaked on NA plates for purification. In order to avoid the selection of CO₂ fixers on MM agar plates (containing above compounds), the plates were placed in a dessicator in which filter soaked in 10% KOH solution was kept for depleting atmospheric CO₂. The purified colonies were rechecked on MM agar plates containing either naphthalene or phenanthrene as the sole carbon source.

The naphthalene- and phenanthrene-degrading organisms were further characterized for their resistance to antibiotics and heavy metal ions. The Gram staining was performed and the organisms were identified by carrying out various biochemical tests according to the descriptions in Bergey's Manual of Systematic Bacteriology (Vol. 1 and Vol. 2; 1986).

Since analysis of cell wall components is one of the most useful techniques for the identification of actinomycetes, the thin-layer chromatography (TLC) using cell hydrolysate was carried out to analyze cell wall amino-acids (Staneck and Roberts, 1974). The dried cells were hydrolyzed with 6N HCl at 100°C for 18h. The hydrolysate was filtered, dried on a rotary evaporator and resuspended in ddH₂O. The sample prepared in this way was loaded onto cellulose TLC sheet (Merck No. 5552). The TLC sheet was run for 3h in a jar using methanol : water : 6N HCL : pyridine (80 : 26 : 4 : 10; v/v) as solvent system. The plate was visualized by spraying it with 0.2% (w/v) ninhydrin solution (prepared in water saturated n-butanol) and heating it at 105°C for 5min. The position of a compound on chromatograph was defined in terms of its Rf value. This was calculated as the ratio of the distance from the origin travelled by the compound to the distance moved by the solvent front.
3.5 GROWTH AND MAINTENANCE OF ORGANISMS

The ability of the organisms to utilize various aromatic compounds was checked by inoculating the test organism either on solid or in liquid MM containing the appropriate aromatic substrate as the sole source of carbon and energy. The growth was assessed for 1-7d depending on the substrate and the organism. The incubation temperature for all the organisms was 30°C except for *E. coli* strains which were incubated at 37°C.

For routine work, wild-type naphthalene- and phenanthrene-degrading organisms were maintained on MM containing either naphthalene or phenanthrene as the case may be. Other organisms were maintained on NA or LB agar plates containing appropriate antibiotic(s) at 4°C up to a month. All the organisms were preserved in 10% (v/v) glycerol stocks at -70°C.

For performing the enzyme assays for either naphthalene- or phenanthrene-degrading pathways, 300ml of MM containing the appropriate carbon source were inoculated with 6ml NB cultures which had been grown overnight with shaking. The flasks were shaken at 180rpm in an incubator shaker. For induction purposes, the potential inducers were added during the early exponential phase of growth of cultures on succinate and the incubation was continued overnight (12-14h).

3.6 PREPARATION OF CELL SUSPENSIONS AND CELL EXTRACTS FOR ENZYME ASSAYS

In order to perform the enzyme assays using whole cells, following growth of cells in liquid cultures (Section 3.5), they were centrifuged in a Sorvall RC-5C centrifuge at 10,000x g for 10min. The cells were washed three times with cold (4°C) 0.1M phosphate buffer (pH 7.4) and were finally resuspended in the same buffer at a final concentration of 0.1g wet weight of cells per ml. Cell suspensions were stored at 4°C and were used within 6h.

In order to prepare the crude cell extracts (CE) for enzyme assays, the cell suspensions were disrupted by passing through an AMINCO French pressure cell.
(AMINCO, SLM instruments Inc., Urbana, Illinois) at 17,000 psi. The cell debris was removed by centrifuging at 27,000xg for 45 min at 4°C. The supernatant (CE) was saved and stored at 4°C. When enzyme assays were to be carried out in the presence of nicotinamide adenine dinucleotide (NAD) or reduced NAD+ (NADH), CE were centrifuged at 100,000xg for 90 min in a Beckman model TL-100 ultracentrifuge at 4°C to remove the particulate fraction rich in NADH oxidase. This supernatant fluid is referred to as ultracentrifuged cell extracts (UE). CE and UE were maintained at 4°C until used for enzyme assays which was normally within 12h of their preparation.

3.7 ENZYME ASSAYS

All spectrophotometric enzyme assays were carried out at 25°C in quartz cuvettes of 1.0 cm path length containing a final volume of 3 ml using a Gilford Spectrophotometer. The change in absorbance was followed for 2 min. Unless stated otherwise, the specific activities were expressed as nmoles of substrate utilized or product formed/min/mg protein.

Naphthalene dioxygenase activity was measured using whole washed cells by the method as described by Shamsuzzaman and Barnsley (1974a). The decrease in absorbance at 276 nm due to disappearance of naphthalene was followed. The extinction coefficient for naphthalene at 276 nm of 4.51 mM⁻¹ cm⁻¹ was used. The specific activity of 1,2-dihydroxynaphthalene oxygenase was measured in a reaction mixture containing CE in 50 mM acetic acid-NaOH buffer (pH 5.5) and 40 mM 1,2-dihydroxynaphthalene. The initial rate of decrease of the absorbance at 285 nm was measured (Shamsuzzaman and Barnsley, 1974b). The specific activity was calculated using an extinction coefficient of 2.13 mM⁻¹ cm⁻¹, the difference between that of 1,2-dihydroxynaphthalene (4.05) and the reaction product (1.92). Salicylaldehyde dehydrogenase activity was determined using UE from the rate of increase in absorbance at 340 nm caused by the reduction of NAD into NADH in a reaction mixture containing 20 mM tetrasodium pyrophosphate-HCl (pH 8.5) buffer, 0.1 ml salicylaldehyde (3 mM aqueous solution of freshly distilled salicylaldehyde) and 0.1 ml NAD (150 mM). The rate was calculated using an extinction coefficient of 3.84 mM⁻¹ cm⁻¹ (Shamsuzzaman and Barnsley, 1974b). The specific activity of salicylate hydroxylase (EC 1.14.13.1) was assayed at 296 nm using UE in a cuvette containing 1 mM EDTA.
(pH 8.0), 133 mM sodium salicylate, 147 mM NADH and 0.02 M phosphate buffer (pH 7.6) in a total volume of 3 ml. The molar extinction coefficient of 4.7 mM⁻¹ cm⁻¹ was used to calculate specific activity (White-Stevens and Kumin, 1972).

The activities of catechol-2,3-dioxygenase (EC 1.13.11.2) and catechol-1,2-dioxygenase (EC 1.13.11.1) were determined using CE according to the procedures essentially described by Murray and Williams (1974). For assaying catechol-2,3-dioxygenase, the CE was heated at 55°C for 2 min to inactivate catechol-1,2-dioxygenase and was centrifuged to remove precipitated protein. Catechol-1,2-dioxygenase was assayed by inactivating catechol-2,3-dioxygenase by incubating CE with 30 mM H₂O₂ for 2 min at room temperature (Murray and Williams, 1974). Gentisate-1,2-dioxygenase (EC 1.13.11.4) was assayed by measuring the rate of formation of maleylpyruvate at 334 nm in a reaction mixture containing 0.15 μmoles of gentisate in 3 ml of 0.1 M phosphate buffer (pH 7.4) (Crawford et al., 1975). The CE was activated by incubation with 2.5 mM ferrous ammonium sulphate for 20 min at 25°C prior to assay.

2-Carboxybenzaldehyde dehydrogenase activity was calculated from the rate of increase in absorbance at 340 nm due to the reduction of NAD in 0.05 M phosphate buffer (pH 7.8) containing 2 mM NAD, 0.033 mM 2-carboxybenzaldehyde and UE (Barnsley, 1983a). The activity of 1-hydroxy-2-naphthoate oxygenase was assayed in 10 mM phosphate buffer (pH 7.4) containing 600 nmoles of 1-hydroxy-2-naphthoate and CE, according to the method described by Kiyohara and Nagao (1977). The CE was activated by incubating with 600 nmoles of ferrous sulphate just prior to assay.

The activities of protocatechuate-4,5-dioxygenase (EC 1.13.11.8) and protocatechuate-3,4-dioxygenase (EC 1.13.11.3) were assayed using CE essentially as described by Ono et al. (1970).

3.8 DETERMINATION OF PROTEIN CONCENTRATION

Protein concentration in CE and UE were determined by the method of Bradford (1976) using the Bio-Rad Protein Assay Kit II. Bovine serum albumin (BSA) was used as standard. The whole cells were treated with trichloroacetic acid (final concentration
5%, w/v) before estimating the protein concentration. The absorbance was read at 595 nm in a Spectronic 21 UVD Spectrophotometer (Milton Roy Company, Rochester, NY).

3.9 ISOLATION AND SELECTION OF MUTANTS

Various procedures were followed in order to isolate different derivatives of the wild-type naphthalene-degrading organism (*P. putida* RKJI) which were unable to utilize either naphthalene (Nap⁻ Sal⁺) or salicylate (Nap⁺ Sal⁻) or both (Nap⁻ Sal⁻).

3.9.1 Growth in Nutrient Broth

Spontaneous mutants were isolated by inoculating 5 ml NB with approximately 10⁶ cells of the organism and the culture was grown overnight with shaking to stationary phase, this represented one cycle. After 10 such cycles, the cultures were serially diluted and plated onto NA plates. The colonies were then picked up and patched onto fresh NA plates and replica-plated onto MM agar plates containing either naphthalene or salicylate as the sole source of carbon and energy.

3.9.2 Treatment with N-Methyl-N'-1-Nitro-N-Nitrosoguanidine (NTG)

Nutrient broth (25 ml) was inoculated with 0.5 ml of overnight NB grown culture and was incubated with shaking till late log phase. The cells were pelleted down by centrifugation, washed twice with 0.1 M citrate buffer (pH 5.5) and resuspended in 50 ml of buffer. The cell suspension (5.0 ml each) was distributed in different tubes. NTG was added to a final concentration of 25 μg/ml to each tube and the tubes were incubated at 30°C. The cultures were withdrawn at different time intervals (5, 10, 15, 20, 30, 60 min), immediately centrifuged and were washed with 0.1 M phosphate buffer (pH 7.4). Serial dilutions were plated onto NA plates to determine the total number of cells survived for each time point. The time interval at which 90-95% of cells were killed was chosen for mutagenizing the cells. After plating out serial dilutions, the colonies were picked up and patched onto fresh NA plates and then replica-plated onto MM agar plates containing either naphthalene or salicylate. The colonies were also replica-plated onto MM agar plates containing glucose in order to avoid auxotrophic
mutants. Different derivatives unable to utilize naphthalene or salicylate were selected for further purification and characterization.

3.9.3 Treatment with Mitomycin C

Approximately $10^6$-$10^7$ cells of overnight NB grown cultures were inoculated in NB in the presence of mitomycin C at concentrations varying from 0-30μg/ml (in 5μg increments) and they were grown with shaking at 30°C for 20h. The minimum inhibitory concentration (MIC) of mitomycin C which inhibited the growth of organisms was determined and the cells were grown at sub-MIC for 48h overnight and then transferred to fresh NB containing mitomycin C at same concentration. This represented one cycle. The cultures were grown for 3 such cycles. The cultures from each cycle were serially diluted and plated onto NA plates. The colonies thus obtained were picked up and patched again and were checked for their phenotypic properties by replica-plating as described above.

3.10 ISOLATION OF REVERTANTS OF MUTANTS

To isolate the revertants of different mutants (such as Nap* Sal* and Nap*Sal*) obtained by above methods, overnight NB grown cultures (10ml) were harvested, washed with 0.1M phosphate buffer (pH 7.4) and resuspended in 1.0ml of MM. One hundred μl of cells were plated onto MM agar plates containing either naphthalene or salicylate as the sole source of carbon and energy. The plates were incubated at 30°C and were observed for 7d. In order to determine the total number of cells plated onto MM agar plates, 100μl of resuspended cells were serially diluted and plated onto NA plates.

3.11 CONJUGATION EXPERIMENTS

Conjugation experiments were performed by either filter matings or plate matings. The filter matings were performed as follows. The donor strains were grown without shaking whereas the recipient strains were grown with shaking at 180rpm in LB or NB containing appropriate antibiotic(s) as required till late log phase. The donor and the recipient cultures were mixed gently by swirling in different ratios (1 : 2.5,
1 : 1, or 2.5 : 1) in 50ml flasks. The cells were collected on a 0.45μm Whatman filter membrane and the filter membranes were placed in NA plates and were incubated at 30°C for 16h. The bacterial growth from the membrane was then resuspended in MM and 0.1ml aliquots of 10^0, 10^1 and 10^2 dilutions were spread onto the appropriate selection media contra-selecting against both the donor and recipient strains. Plate matings were carried out as described by Dunn and Gunsalus (1973) except that donor and recipient strains were grown separately in 10ml NB. In order to determine the frequency of transfer in filter and plate matings, viable counts for donor strains were calculated by plating appropriate dilutions onto NA plates.

Transconjugants obtained on selective media were further purified and characterized. Putative transconjugants were purified on the same selection media and checked for the presence of plasmid(s) by rapid plasmid DNA isolation method (Section 3.12) and by nucleic acid hybridization (Section 3.20), if needed. Some of such transconjugants were retained for future work.

In order to transfer recombinant plasmids from putative clones to the other strains, triparental matings were carried out. This was performed by using helper plasmid RP4 or pRK2013 as described by Ditta et al. (1980). The recombinant plasmids were also mobilized by using the E. coli strain S-17.1 (carries RP4-2 (tra) function integrated into chromosome; Simon et al., 1983). Plate matings were performed by mixing one drop each of overnight NB grown cultures of E. coli strain carrying either RP4 or pRK2013 (Table 3), the donor and the recipient strains on NA plates. Following overnight growth on NA plates at 30°C, the cells were resuspended in MM and appropriate dilutions of mating mixtures were plated onto NA plates containing antibiotic(s) and/or aromatic carbon source contra-selecting against all three strains. Colonies obtained on these plates were purified and characterized as described above.

3.12 PLASMID DNA ISOLATION

In order to check the presence of extrachromosomal or plasmid DNA in various strains, different methods were attempted which are specific for both small and large plasmids. The method described by Portnoy et al. (1981) was followed with minor
modifications. One ml of overnight NB grown cultures was used for minipreparation of plasmid DNA. The cells were harvested and after washing once with TE buffer (pH 8.0) were resuspended in 40μl TE containing 10mg/ml lysozyme. Lysis buffer (TE containing 4% SDS, pH 12.4) was added and mixed immediately by gentle inversion of the tubes. The samples were incubated at 37°C for 20min to ensure complete lysis and were brought to pH 8.0 by adding 30μl of 2M Tris-HCl buffer (pH 7.0). The DNA was precipitated by adding 0.16ml of 5M NaCl and 0.55 volume of cold isopropanol.

The method of Wheatcroft and Williams (1981) was followed except that the reagent A, in which the pelleted cells were resuspended, consisted of TE buffer (pH 8.0) and 2% Tween-80 instead of 5% Antifoam RD emulsion. The cells were lysed by the addition of 1M NaOH saturated with SDS. The cell lysate was directly loaded onto the agarose gel. After 10min, electrophoresis was carried out as described in Section 3.15.

The alkaline lysis method (Birnboim and Doly, 1979) was followed with minor modifications. The cells were incubated in 10mg/ml lysozyme solution for 15min at 37°C before lysing them with SDS. The methods of Hansen and Olson (1978) and Kado and Liu (1981) were followed essentially as described by the authors.

For large scale extraction of plasmid DNA, sucrose density gradient ultracentrifugation was carried out using the method of Wheatcroft and Williams (1981). The sucrose gradients were prepared from a solution of 20% (w/v) sucrose in sterile ddH₂O in 14ml centrifuge tubes. The solution was slowly frozen solid and thawed three times by sequentially transferring them from 0°C to -20°C and then from -20°C to 0°C. Finally, the tubes were stored at -20°C and were thawed overnight at 0°C before using them the next day. The cell lysates were layered onto these sucrose gradients and were centrifuged at 100,000g for 2h at 20°C using a swing-out rotor (TST 41.14) in a Centrikon T-2070 ultracentrifuge (Kontron Instruments, Sweden). After centrifugation, fractions (0.5ml) were collected. Twenty μl samples of these fractions were electrophoresed and the fractions containing the most pure plasmid DNA were pooled together and the DNA was precipitated after dialysing the samples against TE buffer (pH 8.0) to remove the sucrose.
The method which gave satisfactory yields of large plasmids was the one described by Anderson and McKay (1983). For rapid minipreparations of plasmid DNA from various transformants and transconjugants, 2 ml of overnight cultures grown in LB or NB were used. Plasmid DNA isolated this way was used directly for restriction endonuclease digestion. For the preparation of plasmid DNA on large scale, the cells were grown overnight in 500 ml NB. The DNA was purified on cesium chloride-ethidium bromide (CsCl-EtBr) gradients run at 55,000 rpm for 20 h using a fixed angle TFT 80.13 rotor. The plasmid DNA bands obtained were collected under UV light and were extracted with isoamyl alcohol to remove ethidium bromide (Maniatis et al., 1989). Cesium chloride was removed by diluting the DNA solution with three volumes of ddH₂O and adding two volumes of ethanol. The DNA was precipitated by centrifugation at 10,000×g for 15 min. After removing the ethanol and drying the DNA sample, the precipitate was dissolved in appropriate volume of TE buffer (pH 8.0).

For plasmid DNA isolation from *E. coli* strains carrying recombinant plasmids, alkaline lysis method was followed both for rapid analysis and large scale preparations (Birnboim and Doly, 1979). The plasmid DNA was purified on CsCl-EtBr gradients as described above.

### 3.13 ISOLATION OF TOTAL GENOMIC DNA

For the isolation of total genomic DNA for cloning purposes, 100 ml of overnight NB grown cultures were used and the method of Ausubel *et al.* (1988) was followed. The cells were harvested and washed with STE buffer (0.1 M NaCl, 10 mM Tris-HCl and 1 mM EDTA, pH 8.0). The cells were resuspended in 5 ml TE buffer containing 10 mg/ml lysozyme and incubated at 37 °C for 30 min. 0.25 ml of 20% (w/v) SDS and 100 µl of 10 mg/ml pronase were added and mixed thoroughly but gently. The sample was incubated at 30 °C for 1 h. The cell lysate was extracted twice with an equal volume of redistilled phenol (saturated with TE buffer). The aqueous layer was removed carefully and re-extracted with chloroform : isoamyl alcohol (24 : 1, v/v). The DNA in aqueous phase was precipitated by adding 0.1 volume of 3 M sodium acetate (pH 5.2) to give a final concentration of 0.3 M and two volumes of cold (-20 °C) absolute ethanol. The DNA was recovered by spooling with the help of a pasteur pipette that was
bent and sealed in a Bunsen flame. The DNA was then washed with 70% ethanol, dried and dissolved in appropriate volume of TE buffer and stored at 4°C.

3.14 QUANTITATION OF DNA

The concentration of purified DNA was determined using a Gilford Spectrophotometer. Absorbance of appropriately diluted DNA (in TE buffer) was determined at 260nm and 280nm using TE buffer as blank. An absorbance unit of 1.0 at 260nm corresponded to approximate concentration of 50μg/ml of double stranded (ds) DNA (Maniatis et al., 1989). The ratio of absorbance at 260nm : 280nm (O.D.\ _{260nm} : O.D.\ _{280nm}) provided an estimate of the purity of the DNA. The purified DNA preparations generally had O.D.\ _{260nm} : O.D.\ _{280nm} ratio of approximately 1.8 : 1.

3.15 AGAROSE GEL ELECTROPHORESIS

Whole plasmid DNA samples were electrophoresed at nearly 5V/cm in agarose gel using 1X TAE as running buffer. Ethidium bromide was incorporated in the agarose gels at a final concentration of 5μg/ml. The DNA samples were mixed with required volume of 6X gel loading dye (0.25% bromophenol blue in 40% sucrose in ddH₂O) (Maniatis et al., 1989). The samples were loaded onto the gel. The electrophoresis was carried out using horizontal gel electrophoresis apparatus (Pharmacia, USA). DNA bands were visualized on UV transilluminator (Fotodyne, USA) and the gels were photographed through a red filter on a Polaroid (USA) film using a Polaroid camera. Depending on the fragment sizes, endonuclease digests of plasmids were electrophoresed in 0.55-0.7% (w/v) agarose in 1X TAE buffer.

3.16 RESTRICTION ENDONUCLEASE DIGESTION

The restriction endonuclease digestion was carried out by completely digesting the plasmid DNA samples with various restriction enzymes according to the suppliers instructions (Promega Corporation, USA or Boehringer, Mannheim, Germany). The reaction mixture consisted of DNA sample, the enzyme and appropriate amount of corresponding 10X enzyme buffer and the total volume was made up with sterile ddH₂O. Two units of the enzyme were added for every μg of DNA. The sizes of
restriction fragments were determined by electrophoresing DNA samples following complete digestion with different enzymes along with lambda DNA digested with HindIII or EcoRI or HindIII-EcoRI as standard. The molecular sizes of fragments were calculated by plotting the log10 of the number of base pairs versus migration distance (Bearden, 1979).

For the construction of total genomic DNA library, the DNA was suspended in TE buffer at a concentration of 0.5 mg/ml prior to digestion as described by Ausubel et al. (1988). The reaction mixture was set up by adding 50 μl of appropriate 10X restriction enzyme buffer, DNA and ddH₂O to a final volume of 500 μl. Forty ml of the mixture was removed and added to 5 μl of 0.5 M EDTA (pH 8.0) to stop the reaction and keep on ice. To the remaining reaction mixture, the enzyme was added in appropriate units. After every 10 min interval for up to 90 min, 40 μl of the digested sample was removed and 5 μl of 0.5 M EDTA (pH 8.0) was added in a fresh microcentrifuge tube and placed on ice. All the DNA aliquots along with HindIII-digested lambda DNA as standard were electrophoresed in 0.55% (w/v) agarose in 1X TAE buffer. The amount of digestion was assessed. The time point at which majority of DNA fragments were in the range of 20-40 kb size was chosen to scale up the digestion reaction. Following large scale DNA digestion, the digested DNA was extracted with equal volume of equilibrated phenol and chloroform : isoamylalcohol (24:1). The aqueous phase was separated and the DNA was precipitated by adding 0.1 volume of 5 M NaCl and two volumes of absolute ethanol. Following the removal of alcohol and vacuum drying of DNA, it was resuspended in TE buffer and analyzed on a 0.55% (w/v) agarose gel.

3.17 PURIFICATION OF DNA FROM AGAROSE GELS

The DNA fragment(s) were purified from agarose gels as described below. After gel electrophoresis and visualisation of DNA bands under UV light, the DNA fragments of interest were located and cut out from the agarose gel. The agarose slices were placed in a preweighed eppendorf tube. DNA fragments below 10 kb were eluted from the agarose gels onto silica matrix using a Gene Clean Kit (BIO 101 Inc., USA) according to the method of Vogelstein and Gillespie (1979). To purify larger DNA fragments, electrophoresis was carried out in 0.6% low melting temperature agarose
The DNA band of interest was located, cut out and transferred to a preweighed tube. Five volumes of TE buffer were added to the agarose slice and incubated at 65°C to melt the gel. An equal volume of phenol equilibrated with TE buffer was added to the solution and mixed thoroughly. The aqueous phase was re-extracted once with phenol : chloroform (1:1) and once with chloroform : isoamyl alcohol (23:1). To the aqueous phase, 0.2 volume of 10M ammonium acetate and two volumes of ethanol were added. The DNA was recovered by centrifugation at 10,000rpm for 15min and finally resuspended in appropriate volume of TE buffer. The purified DNA was directly used for restriction endonuclease digestion and cloning experiments as needed.

3.18 CLONING EXPERIMENT (DEPHOSPHORYLATION AND LIGATION)

For cloning purposes, dephosphorylation of the vector DNA was carried out using calf intestine alkaline phosphatase (CIAP) (Promega Corporation, USA) according to the suppliers' instructions. This was required in order to minimize self-ligation of vector DNA. The vector DNA (approximately 1μg) digested with appropriate enzyme was treated with one unit of CIAP at 37°C for 2h. The enzyme was inactivated by heating at 65°C for 10min.

For ligation experiments, dephosphorylated vector DNA and the digested foreign DNA were separately extracted with phenol and then with chloroform : isoamyl alcohol. The DNA were precipitated with ethanol and resuspended in appropriate volume of TE buffer (Maniatis et al., 1989). Vector and foreign DNA were mixed in an equimolar ends ratio in a total reaction volume of 20μl containing 2μl of 10X ligation buffer and 2units of T4 DNA ligase (Promega Corporation, USA). The reaction mixture was incubated at 16°C for 20h (Maniatis et al., 1989). Ligation mixture was then heated at 65°C for 5min to inactivate T4 DNA ligase. The composite plasmid DNA molecules were precipitated by adding 0.1 volume of 3M sodium acetate (pH 5.2) and two volumes of absolute ethanol and finally resuspended in 10μl TE which was then used for transformation/electroporation.
3.19 TRANSFORMATION OF RECOMBINANT PLASMID DNA

Two different methods of transformation were employed in order to transform different bacterial strains with recombinant plasmid DNA molecules.

3.19.1 Transformation of Plasmid DNA

The competent cells for transformation were prepared as described by Maniatis et al. (1989) with minor modifications. Luria broth (100ml) was inoculated with overnight grown culture of *E. coli* strains and incubated at 37°C for 3-4h with vigorous shaking until the cells had reached in mid-log phase. The cells were recovered by centrifugation at 4,000rpm for 10min at 4°C. They were then washed two times with 50ml ice-cold 0.1M CaCl₂ and resuspended in 4ml ice-cold 0.1M CaCl₂. The cells were dispensed into aliquots of 200μl each in eppendorf tubes on ice and were stored at -70°C for not more than one month.

Transformation was carried out by mixing an appropriate amount of DNA and stored on ice for 30min before giving heat shock at 42°C for 90sec (Maniatis et al., 1989). The tubes were rapidly transferred to an ice bath and 800μl LB medium was added. The cultures were incubated for 1h at 37°C to allow the bacteria to recover and to express the antibiotic resistance marker encoded by the plasmid before plating onto LB agar plates containing the appropriate antibiotic(s).

3.19.2 Electroporation

In order to obtain a better frequency of transformation, electroporation experiments were also performed. Electrocompetent cells from *E. coli* and *P. putida* strains were prepared according to the method of Dower et al. (1988) with minor modifications. Superbroth (Trypton 9.6g, Yeast extract 6g, NaCl 1.5g in 300ml ddH₂O) was inoculated with an overnight LB grown culture and was incubated with vigorous shaking upto an O.D₁₆₀₀nm of 0.5. The cells were harvested at 4°C and washed twice with 100ml of ice-cold 1mM HEPES (N-[2-hydroxyethyl]piperazine-N’-[2-ethanesulfonic acid) buffer and once with 20ml ice-cold 10% (v/v) glycerol. The pellet was then resuspended in 1ml of 10% glycerol. Aliquots of 100μl each in eppendorf
tubes were stored at -70°C. The cells were thawed in ice bath just prior to electroporation.

For electroporation, the cells were mixed with 500ng to 1µg DNA and placed in an ice-cold 0.4cm electroporation cuvette (Bio-Rad laboratories, USA). The electric pulse was given at 2.5µfd and 800 ohms using Gene Pulsar apparatus (Bio-Rad Lab., USA). One ml of LB was immediately added, mixed thoroughly and incubated at optimal temperature for 1h for gene expression before plating out onto LB agar plates containing appropriate antibiotic(s). Whenever vector(s) with β-galactosidase gene(s) was used for cloning experiments, the selection medium contained β-D-isopropyl-thio-galactopyranoside (IPTG) and 5-bromo-4-chloro-3-indolyl-β-galactopyranoside (X-Gal).

Since the cells of *Moraxella* sp. and *A. sulphureus* were lysed in 1mM HEPES while carrying out the electroporation experiments, they were washed only with 10% glycerol two times. Also, in these two cases, electroporations were carried out using different cuvettes (0.1, 0.2 and 0.4cm) and varying the resistance from 200 to 1,000ohms and the voltage applied (1.5kV or 2.5kV). The cells were expressed in NB for 2h before plating out onto either NA plates supplemented with appropriate antibiotic(s) or selective MM agar plates.

3.20 NUCLEIC ACID HYBRIDIZATION

Hybridization experiments were carried out in order to locate the homologous sequences. The DNA was immobilized on to the Hybond-N nylon membrane (Amersham International plc., Amersham, UK) either by *in situ* lysis of bacterial colonies (Maniatis *et al.*, 1989) or by Southern blotting (Southern, 1975). Such DNA noncovalently attached to the membrane was then hybridized to appropriate [³²P]-labelled probe DNA.

3.20.1 Transfer, Lysis of Colonies and Binding of DNA

For colony hybridizations, individual bacterial colonies of interest were grown on agar plates. The correct membrane size was selected and placed onto the agar
surface. The colonies were then transferred basically according to the method of Maniatis et al. (1989). The membrane was removed from the plate after 1 min and placed with colony side up onto 3MM Whatman paper saturated with 10% (w/v) solution of SDS for 5 min. The membrane was transferred to the second sheet of 3MM paper saturated with denaturing solution (0.5N NaOH, 1.5M NaCl) and left for 5 min. The filter was then transferred to the third sheet of 3MM Whatman paper saturated with neutralization buffer (1.5M NaCl, 0.5M Tris-HCl, pH 7.4 and 1 mM EDTA, pH 8.0). After 5 min, the membrane was washed in 2X SSC and laid on a dry filter paper for drying for 1 h at room temperature. The DNA was then fixed onto the membrane by exposing the DNA side of the membrane to UV light on a transilluminator for 2-5 min. The filters were then sealed inside a heat sealable plastic bag and used for hybridization using appropriate [32P]-labelled DNA probe as described in Section 3.20.4.

### 3.20.2 Southern Blotting (DNA Capillary Transfer)

DNA from agarose gels was transferred onto the nylon membranes essentially according to the procedures described by the manufacturers. After agarose gel electrophoresis, the DNA was depurinated by soaking the gel in 0.25N HCl for 20 min and was denatured in denaturing solution for 1 h at room temperature. The gel was then neutralized by transferring it in neutralization buffer for 30 min at room temperature with gentle shaking. This step was repeated and the gel was washed in 10X SSC before setting up a capillary blot. After blotting, the membrane was washed briefly in 2X SSC to remove any adhering agarose pieces. The DNA was then fixed as described earlier (Section 3.20.1) and the dried membrane containing the fixed DNA was sealed inside a heat sealable plastic bag.

### 3.20.3 Nick-Translation of the DNA

Various DNA fragments to be used as DNA probes were labelled with [α-32P]-dCTP using a Nick Translation Kit (Promega Corporation, USA) according to the supplier's instructions. The reaction mixture contained DNA, 5 μl of 10X nick translation buffer, unlabelled nucleotide mix containing dATP, dTTP and dGTP, [α-32P]-dCTP and enzyme mix in a total volume of 50 μl. The reaction was carried out
at 15°C for 60min and was stopped by adding 5μl of 0.5M EDTA (pH 8.0). The nick-translated DNA was separated from unincorporated nucleotides by size exclusion chromatography on a Sephadex G-50 spin column which was prepared in a 1.5ml eppendorf tube (Maniatis et al., 1989). An aliquot of labelled DNA was added to 5ml aqueous scintillation fluid (2,5-diphenyloxazole (PPO) 4g, 1,4-Bis(5-phenyloxazol-2-yl)benzene (POPOP) 50mg in 1000ml scintillation grade toluene) and amount of radioactivity was determined in a Beta-liquid scintillation counter (Rackbeta 1211, LKB, Sweden). The percent of label incorporated was determined by DE81 filter binding assay (Maniatis et al., 1989). The specific activity of the labelled DNA was calculated as follows:

\[
\text{% incorporation} = \frac{\text{cpm incorporated}}{\text{total cpm}} \times 100
\]

\[
\text{specific activity} = \frac{\text{cpm incorporated} \times \text{dilution factor}}{\text{mg input DNA}}
\]

The [\(^{32}\)P]-labelled DNA probe was denatured in a boiling water bath for 10min and cooled rapidly in ice just before adding to the hybridization buffer.

### 3.20.4 Hybridization of DNA

A corner of the plastic bag containing the membrane (obtained by either colony hybridization or Southern hybridization) was cut and prehybridization buffer (6X SSC, 5X Denhardt's reagent, 0.5% SDS, 100μg/ml Salmon sperm DNA and 50% formamide) was added (Maniatis et al., 1989). The bag was sealed and incubated at 42°C for 2h. The prehybridization solution was removed and same buffer containing [\(^{32}\)P]-labelled probe was added. Hybridization was carried out at 42°C for 16-20h with gentle agitation. At the completion of hybridization, the membranes were removed from the bag and were rinsed in 2X SSC to remove unbound labelled DNA and washed two times by vigorous agitation at room temperature for 15min in each of 2X SSC containing 0.1% SDS and 0.5X SSC containing 0.1% SDS, respectively. Finally, the membranes were washed in 0.1X SSC containing 0.1% SDS at 60°C for 15min. Excess buffer was drained off and the membranes were enclosed in a sealable plastic bag and exposed to X-ray films (Hindustan Photo Films,
India) with intensifying screens at -70°C for varying time periods for up to 3d depending on the brightness of the signals. After exposure, the X-ray films were developed according to the manufacturer’s instructions. The films were developed at room temperature in an X-ray developer for 2-5 min and were washed in 3% acetic acid stop bath for 2 min. The films were then transferred to the fixer solution for 5 min and were washed thoroughly in running tap water for 15 min. The films were air dried before analyzing the autoradiograms.

3.21 MONITORING THE STABILITY OF RECOMBINANT PLASMID

The stability of the recombinant plasmid pRKJ3 in *P. putida* RKJ15 (Nap⁺ Sal⁺ Rif⁺; Table 3) in selective and non-selective conditions was monitored. In case of non-selective conditions, the organism was inoculated in 100 ml NB to an O.D.₆₀₀nm of 0.02. The culture was incubated at 30°C with shaking for 24 h and transferred to 100 ml of fresh NB to obtain the same O.D.₆₀₀nm of 0.02. This represented one cycle of transfer. This was repeated for up to 7 d. Different dilutions of the cultures from each cycle were plated onto NA plates containing Rif (100 µg/ml) to obtain single colonies. After overnight growth, a total of 200 colonies from each cycle were picked up and patched onto NA plates containing Tet (15 µg/ml) and MM agar plates containing either naphthalene or salicylate as the sole carbon source. The number of Tet⁺, Nap⁺, Sal⁺ colonies versus number of days were plotted. Similarly, in case of selective conditions, the stability of pRKJ3 was monitored by inoculating RKJ15 in 100 ml NB containing Tet (15 µg/ml) and carrying out 7 cycles of transfer. A total of 100 colonies obtained after each cycle were screened for Tet⁺ phenotype. The number of Tet⁺ colonies was plotted versus number of days.

For calculation of the generation time of RKJ15 in NB, overnight NB grown culture was inoculated in 250 ml of fresh NB to an O.D.₆₀₀nm of 0.01. The O.D.₆₀₀nm was measured regularly every hour. At each time interval, different dilutions of the liquid culture were also plated onto NA plates in order to calculate the viable cells. This was done till stationary phase of the culture was obtained. Colonies formed for each dilution were counted after overnight growth at 30°C. Time versus O.D.₆₀₀nm or colony forming units/ml (cfu/ml) were plotted.
3.22 CHEMICALS

Naphthalene, phenanthrene, salicylate, o-phthalate, protocatechuate, catechol, 1-hydroxy-2-naphthoate and salicylaldehyde were purchased from Sigma Chemical Company, St. Louis, USA. 2-Carboxybenzaldehyde and 1,2-dihydroxynaphthalene were purchased from Aldrich Chemical Co., Inc., USA.

Mitomycin C, lysozyme, bromophenol blue, formamide, ethidium bromide, pronase, RNase A and all the antibiotics were obtained from Sigma Chemical Co., USA. NTG was obtained from Fluka chemie AG, Switzerland. IPTG, X-Gal, restriction endonucleases, T4 DNA ligase, CIAP, and lambda DNA were purchased from Promega Corporation, USA. All other chemicals used were of analytical grade and were obtained from indigenous commercial sources.