“What day of the month is it?” he said.

Alice considered a little, and then said, “the fourth.”

“Two days wrong,” sighed the Hatter. “I told you butter wouldn’t suit the works,” he added, looking angrily at the March Hare. “It was the best butter,” the March Hare meekly replied.
CHAPTER 2

Materials and Methods

2.1 Chemicals

Agarose, ethidium bromide, X-gal, IPTG, SDS, BSA, acrylamide, bis-acrylamide, β-mercaptoethanol, ammonium persulphate, TEMED, Tris, EDTA, and MOPS were procured from various vendors including Gibco-BRL, Sigma, Calbiochem and others. Tryptone, yeast extract, peptone and agar powder for preparation of culture media were obtained from Hi Media (India). All restriction enzymes, Klenow polymerase, T4 DNA ligase, and polynucleotide kinase were purchased from New England Biolabs Inc. (USA). Lysozyme, DNase I, RNase, Proteinase K were purchased from Roche (Germany). Taq DNA polymerase, DNA and protein molecular weight markers were purchased from Bangalore Genei (India). DNA and RNA purifying kits were purchased from QIAGEN and Promega. Phage packaging kit was from Stratagene (USA). The DNA random primer kit for labelling DNA, and radiolabelled nucleotides were from BRIT (India). Sephadex G-50 and G-25 were from Pharmacia Biotech (Sweden). Custom made oligonucleotides were obtained either from Oswel (UK) or from in-house oligo synthesis facility. Nitrocellulose, Hybond™-N+ and PVDF™ membranes were from Amersham (UK) and Whatman filter paper from Whatman International ltd. (USA). X-ray films were obtained from Konica Corporation (Japan). All other chemicals were purchased from local manufacturers and were of analytical grade.

2.2 Software

All sequence analyses were performed over WWW using SeWeR (Basu 2001) interface. In some special cases, custom-made Perl scripts were used. Other specific softwares are mentioned in this text as and when required.
1 Materials and Methods

2.3 Oligonucleotides

The oligonucleotides used in this study are listed in Table 2.1.

2.4 Plasmids and cosmid

pBluescriptII KS+ (Stratagene) is a high-copy-number, ColE1-based vector, which confers ampicillin resistance (Amp<sub>+</sub>) to the host cell. It also carries a multiple cloning site region in lacZ<sub>α</sub> fragment enabling blue-white screening of the recombinant clone.

pUC19 (Pharmacia) is a high-copy-number, ColE1-based vector containing Amp<sub>+</sub> marker, and carries lacZ<sub>α</sub> fragment enabling blue-white screening of the recombinant clone.

pDB18R (Fujita et al. 1994) is a high-copy-number plasmid construct derived from pTZ18R (Pharmacia). It carries a 1.8 kb KpnI-HindIII fragment containing \( P. \) aeruginosa rpoS promoter and structural gene.

pASB3 (Tanaka and Takahashi 1991) is plasmid pTZ18R carrying a 2.3 kb PstI DNA fragment containing rpoD of \( P. \) aeruginosa.

pLAFR3 (Staskawicz et al. 1987) is a broad-host-range cosmid with Tc<sup>+</sup> marker; allows blue-white screening because of the presence of lacZ<sub>α</sub> fragment.
Materials and Methods 2.4 Plasmids and cosmid

pGEMD (Igarashi and Ishihama 1991) is derived from pGEMEX1 (Promega) containing a 2.1 kb HindIII fragment spanning the full-length rpoD of E. coli.

pGEMAX185 (Igarashi and Ishihama 1991) is derived from pGEMEX1 (Promega) containing 1.2 kb XbaI fragment spanning the full-length rpoA of E. coli.

pGEMBC (Igarashi and Ishihama 1991) is derived from pGEMEX1 (Promega) containing a 10.2 kb HindIII fragment spanning the full-length rpoB, and rpoC gene of E. coli with upstream rplL.

PGEM-T (Promega) is a TA cloning vector of size 3003 bp, and is used to clone PCR product with A-overhang. It contains Amp\(^+\) marker and lacZ\(^{a}\) for screening of recombinant clones.

pCL1920 (Lerner and Inouye 1990) is a low-copy-number vector with pSC101 replicon (~5 copies/cell). It carries streptomycin/spectinomycin resistance marker (encoded by aadA), and also carries lacZ\(^{a}\) that allows blue-white screening of the recombinants.

p4A4 is pBluescriptII KS\(^+\) containing a Sall–PstI insert of ~2 kb containing C-terminal half of rpoD homolog of P. syringae (Lz4W).

p4C12 is pBluescriptII KS\(^+\) containing a ~2.1 kb PstI fragment with the full-length rpoS of P. syringae (Lz4W).

pRPOH5 (Aramaki and Fujita 1996) is plasmid pTZ18R containing a 1.9 kb Sall–PstI fragment spanning the full-length rpoH of P. aeruginosa.

pGL10 (Bidle and Bartlett 1999) is a broad-host-range cloning vector with IncP replicon. It has a genotype, tra\(^-\) mob\(^+\) and Kmr.

pGLSIGS is pGL10 containing the insert of p4C12.

pXRPOS is pGL10 containing PstI–BamHI fragment from p4C12 and BamHI–HindIII fragment from pDB18R, ligated in tandem, which results in a chimeric rpoS.

pME3088 (Schnider-Keel et al. 2000) is a suicide vector with ColE1 replicon with genotype, RK2-Mob Tc\(^+\).

pMERPOS\(^+\) is pME3088 containing a ~750 bp internal fragment of rpoS gene of Lz4W.
2.5 Culture media

All media were prepared using water purified through MilliQ water purification system (Millipore).

1. Culture media for *P. syringae* (Lz4W)

Antractic Bacteria Medium (ABM) broth:

- Peptone 5 g
- Yeast extract 2.5 g
- Water to 1000 ml
- pH adjusted to 7.0-7.2 with 1 M NaOH.
- Sterilized by autoclaving.

**ABM agar**:  
- ABM 100 ml  
- Agar 1.5 g

2. Culture media for *E. coli*

**LB medium**:  
- Tryptone 10 g  
- Yeast extract 5 g  
- NaCl 10 g  
- Water to 1000 ml  
- pH adjusted to 7.0-7.2 with 1 M NaOH.  
- Sterilized by autoclaving.

**LB agar**:  
- LB medium with 1.5% (w/v) agar.

**LB soft agar**:  
- LB medium with 0.6% (w/v) agar.

**SOB medium**:  
- Tryptone 20 g  
- Yeast extract 5 g  
- NaCl 0.5 g  
- Water to 1000 ml  
- pH adjusted to 7. Sterilized by autoclaving, and just before use, 5 ml of sterile 2 M MgCl₂ was added.

**SOC medium**:  
- SOB medium 1000 ml  
- Glucose (1 M) 20 ml

**Z broth**:  
- LB medium supplemented with 0.5% (w/v) CaCl₂ (0.5 M).

**Z agar**:  
- Z broth with 0.75% (w/v) agar.
## 2.6 Buffers and solutions

1. **Plasmid isolation solution for alkaline lysis**

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

   **Solution 2**: NaOH 0.2 M  
   SDS 1%  

   **Solution 3**: Potassium acetate (5 M) 60 ml  
   Glacial acetic acid 11.5 ml  
   Water 28.5 ml  
   The pH of the solution is approximately 4.8.

2. **Electrophoretic buffer for nucleic acids**

   **TAE**:  
   Tris-acetate 40 mM  
   EDTA 2 mM  
   Prepared as 50 × concentrated stock solution and used as 0.5 × concentration.  

   **TBE**:  
   Tris-borate 90 mM  
   EDTA 2 mM  
   Prepared as 10 × stock solution and used as 0.5–1 × concentration.

3. **Hybridization solution**

   Na$_2$HPO$_4$ 0.5 M  
   SDS 7%

4. **Buffers for transformation**

   **TB**:  
   PIPES (pH 6.7) 10 mM  
   CaCl$_2$·2H$_2$O 15 mM  
   KCl 250 mM  
   MnCl$_2$ 55 mM  

   **RF1**:  
   RbCl 100 mM  
   MnCl$_2$·4H$_2$O 50 mM  
   potassium acetate 30 mM  
   CaCl$_2$·2H$_2$O 10 mM
Materials and Methods

2.6 Buffers and solutions

Glycerol 15% v/v
pH adjusted to 5.8 by glacial acetic acid, filter-sterilized and stored frozen at -20°C.

RF2:

MOPS 10 mM
CaCl₂·2H₂O 75 mM
RbCl 10 mM
Glycerol 15% v/v
pH adjusted to 6.5 by adding 1 M KOH, filter-sterilized and stored frozen at -20°C.

5. Native polyacrylamide gel (10%)

For 10 ml—

- 30% acrylamide mix 3.3 ml
- 1.5 M Tris (pH 8.8) 2.5 ml
- APS (10%) 100 μl
- TEMED 4 μl
- water 4.1 ml

The gel was run in buffer containing 25 mM Tris (pH 8.3), 250 mM glycine.

6. Reagents for SDS-PAGE

Resolving gel (10%): For 10 ml—

- water 4 ml
- 30% acrylamide mix 3.3 ml
- 1.5 M Tris (pH 8.8) 2.5 ml
- SDS (10%) 100 μl
- APS (10%) 100 μl
- TEMED 4 μl

Stacking gel (5%): For 10 ml—

- water 6.8 ml
- 30% acrylamide mix 1.7 ml
- 1.0 M Tris (pH 6.8) 1.25 ml
- SDS (10%) 100 μl
- APS (10%) 100 μl
- TEMED 10 μl

Running buffer: Tris (pH 8.3) 25 mM
Glycine 250 mM
SDS 0.1%
Materials and Methods

1 \times \text{Sample buffer}: \begin{align*}
\text{Tris (pH 6.8)} & : 50 \text{ mM} \\
\text{Dithiothreitol} & : 100 \text{ mM} \\
\text{SDS} & : 2\% \\
\text{Bromophenol blue} & : 0.1\% \\
\text{Glycerol} & : 10\%
\end{align*}

7. Buffers and solutions for immunoblotting

Transfer buffer for semi-dry transfer of proteins:

\begin{align*}
\text{Glycine} & : 39 \text{ mM} \\
\text{Tris} & : 48 \text{ mM} \\
\text{SDS} & : 0.037\% \\
\text{Methanol} & : 20\%
\end{align*}

\text{TBS:}

\begin{align*}
\text{Tris-HCl (pH 7.5)} & : 10 \text{ mM} \\
\text{NaCl} & : 150 \text{ mM}
\end{align*}

Made as 10 \times \text{ stock.}

\text{TBS-T:}

TBS with 0.1\% \text{ Tween-20}^a

Alkaline phosphatase (AP) buffer:

\begin{align*}
\text{Tris-Cl (pH 9.5)} & : 100 \text{ mM} \\
\text{NaCl} & : 100 \text{ mM} \\
\text{MgCl}_2 & : 5 \text{ mM}
\end{align*}

8. Buffers for phage handling

\text{SM buffer:}

For 1000 ml buffer:

\begin{align*}
\text{NaCl} & : 5.5 \text{ g} \\
\text{MgSO}_4 \cdot 7\text{H}_2\text{O} & : 2.0 \text{ g} \\
1 \text{ M Tris-HCl (pH 7.5)} & : 50 \text{ ml} \\
\text{Gelatin (2\% \text{ w/v})} & : 50 \text{ ml} \\
\text{Water to 1 liter. Sterilized by autoclaving.}
\end{align*}

\text{Citrate buffer:}

\begin{align*}
\text{Citric acid (0.1 M)} & : 4.7 \text{ volumes} \\
\text{Sodium citrate (0.1 M)} & : 15.4 \text{ volumes}
\end{align*}

9. Other buffers

\text{TE:}

\begin{align*}
\text{Tris-Cl (pH 8.0)} & : 10 \text{ mM} \\
\text{EDTA} & : 1 \text{ mM}
\end{align*}

\text{20} \times \text{ SSC:}

\text{NaCl} : 173.5 \text{ g}

^a\text{ Tween is a registered trademark of ICI Americas Inc.}
2.7 Bacterial strains

*E. coli* and *P. syringae* strains used in this study are listed in Table 2.2.

2.8 Antibiotics

Antibiotics used in this study and their concentrations are shown in Table 2.3.

2.9 Culturing of bacteria

Typically, the growth measurement was carried out by monitoring the turbidity of the culture, measured as optical density (OD) at 600 nm, in rich medium (LB for *E. coli*; ABM for *P. syringae*). For growth comparison of different strains, pre-inoculum of bacteria from the same stages of growth was diluted 1000 times in 100–150 ml medium, taken in 500 ml conical flask. The flasks were continuously shaken at 200 rpm at the required temperature (22°C or 4°C for Lz4W). The samples were withdrawn at regular interval and OD$_{600}$ was measured either undiluted or by diluting the culture 5 times with water. In the latter case, absorbance was calculated by multiplying the OD obtained by the dilution factor. All the measured samples were measured either diluted or undiluted throughout the measurement. The undiluted culture OD$_{600}$ maximum was 1.8 for Lz4W, which was equivalent to OD$_{600}$ 3 for diluted samples.

2.10 Preparation of crude cell extract

Cells were grown to required growth phase and harvested. The cell pellet was then resuspended in phosphate buffer (50 mM potassium phosphate, pH 7.0; 5 mM EDTA; 10% glycerol; 25 μM phenylmethylsulfonyl fluoride) to an estimated OD of 10. Cells were then sonicated with Branson W-250 sonicator on ice. Typically cells were given four rounds of five 2 s pulses with constant duty cycles and microtip settings of 2–3. Debris was pelleted by centrifugation at 4°C for 10 minutes at 12,000 g. The supernatant was collected in a separate tube and stored at -70°C.
### TABLE 2.2 Bacterial strains used in this study

<table>
<thead>
<tr>
<th>Strains</th>
<th>Genotype/Description</th>
<th>Source/Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Escherichia coli strains</strong>&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DH5α</td>
<td>Δ(argF-lac)U169 supE44 hsdR17 recA1 endA1 gyrA96 thi-1 relA1 φ80dlacZΔM15</td>
<td>Hanahan (1983)</td>
</tr>
<tr>
<td>DH10B&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Δ(mrr hsdRMS mcrBC) mcrA ΔlacX74 deoR recA1 endA1 araD139 φ80dlacZ ΔM15 Δ(ara, leu)7697 galU galK rpsL nupG</td>
<td>Grant et al. (1990)</td>
</tr>
<tr>
<td>MC4100</td>
<td>Δ(argF-lac)U169 rpsL150 relA1 araD139 flb5301 deoC1 ptsF25</td>
<td>Casadaban (1976)</td>
</tr>
<tr>
<td>RH90</td>
<td>MC4100 rpoS359::Tn10</td>
<td>Barth et al. (1995)</td>
</tr>
<tr>
<td>RH100&lt;sup&gt;c&lt;/sup&gt;</td>
<td>MC4100 Δ(nlpD–rpoS)360 zgc-3251::Tn10</td>
<td>Hengge-Aronis et al. (1993)</td>
</tr>
<tr>
<td>GJ2733</td>
<td>MC4100 csiD::lac rpoS359::Tn10</td>
<td>Rajkumari and Gowrishankar (2001)</td>
</tr>
<tr>
<td>GJ2734</td>
<td>MC4100 osmY::lac rpoS359::Tn10</td>
<td>Rajkumari and Gowrishankar (2001)</td>
</tr>
<tr>
<td>GJ2782</td>
<td>RH100 ara&lt;sup&gt;+&lt;/sup&gt; [A katE::lac(Km)]</td>
<td>Rajkumari and Gowrishankar (2002)</td>
</tr>
<tr>
<td>JM101</td>
<td>supE44 thi Δ(gpt-lac)5 F'[traD36 proAB&lt;sup&gt;+&lt;/sup&gt; lacI&lt;sup&gt;+&lt;/sup&gt; lacZΔM15]</td>
<td>Messing (1979)</td>
</tr>
<tr>
<td>MJMRH</td>
<td>JM101 rpoS::Tn10</td>
<td>This study.</td>
</tr>
<tr>
<td>S17-1</td>
<td>pro recA1 RP4-2 integrated (Tc::Mu) (Km::Tn7) (Sm&lt;sup&gt;+&lt;/sup&gt;Tp&lt;sup&gt;+&lt;/sup&gt;)</td>
<td>Simon et al. (1983)</td>
</tr>
<tr>
<td>ZK918</td>
<td>W3110 ΔlacU169 tna-2 AMAV103 rpoS::kan</td>
<td>Bohannon et al. (1991)</td>
</tr>
<tr>
<td>UQ285</td>
<td>P90A5 lacZ4 Lam&lt;sup&gt;+&lt;/sup&gt; rpoD285(ts) argG75</td>
<td>Isaksson et al. (1977)</td>
</tr>
<tr>
<td><strong>Pseudomonas syringae strains</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lz4W</td>
<td>Natural isolate from Antarctica</td>
<td>Shivaji et al. (1989)</td>
</tr>
<tr>
<td>MBLz4W</td>
<td>Lz4W rpoS::pME3088 (Tet&lt;sup&gt;+&lt;/sup&gt;)</td>
<td>This study.</td>
</tr>
</tbody>
</table>

<sup>a</sup> All the strains are F<sup>−</sup> unless stated.

<sup>b</sup> DH10B is a trademark of Invitrogen corporation.

<sup>c</sup> The Tn10 allele in RH100 was originally designated zfi-3251::Tn10, based on the calibration in an earlier edition of the *E. coli* K-12 linkage map.
**2.11 Protein estimation**

The concentration of the protein in the crude cell extract were determined by Bradford method (Bradford 1976) by BIO-RAD dye-reagent™ according to the supplier’s protocol.

**2.12 Enzyme assays**

**2.12.1 β-galactosidase assay**

Assays for determination of β-galactosidase enzyme activity in cultures were performed as described in Miller (1992) and the activity values are calculated in Miller units, as described therein.

**2.12.2 Catalase assay**

**2.12.2.1 Hydrogen peroxide bubbling test**

Quick scoring for catalase phenotype was done by placing a drop of H₂O₂ (30% v/v) directly on the bacterial colony as mentioned in Mulvey et al. (1988). The rate of bubble formation was taken as measure of catalase activity.

**2.12.2.2 Spectrophotometric assay**

Total catalase activity measurement was carried out as described in Visick and Clarke (1997), which was based on Beers and Sizer (1951). Briefly, 25 μl of the crude extract was diluted to 1.5 ml with 50 mM potassium phosphate buffer, pH 7.0. Then 2.5 μl of 30% H₂O₂ was added and the absorbance of the samples at 240 nm was measured every 15 s for 1 min. The specific activity of the catalase (μmol of H₂O₂ decomposed/min/mg of total protein) was then calculated as follows:

\[
\frac{1,000 \times \Delta A_{240} \text{ / min}}{43.6 \times mg \text{ of protein/ml of reaction mixture}}
\]
2.13 Activity staining for catalase

Negative staining of catalase activity on native gel was carried out as described previously (Gregory and Fridovich 1974). Although the same group published an improved protocol later in Clare et al. (1984), we found the older protocol gives consistent results.

In this method, the crude cell extract (Section 2.10) was separated on 8–10% nondenaturing polyacrylamide gel (see Section 2.6) with no stacking gel. The glycerol in the extraction buffer (Section 2.10) was enough to load the samples in the gel. A separate well was generally used to load a mixture of bromophenol blue in 20% glycerol to follow the migration. The gel was run at constant current (20 mA) for required amount of time, at 4°C. After the run, the gel was soaked in diaminobenzidine (0.5 mg/ml), horseradish peroxidase (50 μg/ml) in 50 mM potassium phosphate (pH 7.0) for 1 h at room temperature. The gel was then rinsed and soaked in 20 mM H₂O₂ in phosphate buffer until staining was complete. The catalase bands appears as unstained white bands on dark background.

2.14 Genetic techniques

2.14.1 Bacterial conjugation

Plasmids were mobilized to Lz4W by biparental mating between Lz4W and E. coli S17-1, transformed with the suitable plasmid. The donor (S17-1, transformed with suitable plasmid) and the recipient (Lz4W) strains were grown in 3 ml of appropriate medium supplemented with required antibiotics to mid-logarithmic phase (OD₆₀₀ 0.6–0.9). The cells were pelleted by centrifugation at 6,000 rpm for 5 min at 4°C, and were washed with 1.5 ml of sterile ABM or LB broth. The cell pellets were resuspended in 100 μl of ABM or LB, and the donor and the recipient were mixed in the ratio of 1:5 (v/v). From this mixture, 50 μl was spotted onto Hybond™ N+ membrane (Amersham Life Sciences, Buckinghamshire, UK) placed on ABM agar. Following incubation for 24–72 h at 22°C, the cells were scraped off from the membrane with sterile toothpicks, re-suspended in ABM, and appropriate dilutions were plated on selection media. The plates were incubated at 22°C for 48 h to obtain exconjugants.

2.14.2 P1 lysate preparation on RH90

From an of overnight culture of the strain RH90 (see Table 2.2) 0.3 ml in Z broth was mixed with 10⁷ plaque forming units (pfu) of a stock P1 lysate prepared on strain MG1655. Adsorption was allowed to occur at 37°C for 20 min and the lysate was prepared in the following way.
To 0.3 ml of the infection mixture, 10 ml of Z broth was added and incubated at 37 °C with slow shaking until growth followed by visible lysis of the culture occurred (4–6 h). The lysate was treated with 0.3 ml of chloroform, centrifuged and the clear lysate was stored at 4 °C with chloroform.

To measure the titer of the P1 phage in the lysate, titration was done using a P1-sensitive indicator strain such as MG1655. Aliquots of 100 µl each of serial dilutions (typically 10^5–10^6) were mixed with 100 µl of the fresh culture grown in Z broth. After 15 min adsorption at 37 °C without shaking, each mixture was added into soft agar, overlayed on the Z agar plates, and incubated overnight at 37 °C.

2.14.3 P1 transduction of JM101

To 2 ml of the fresh overnight culture of JM101, grown in Z broth, 10^8 pfu of P1 lysate was added and the mixture was incubated at 37 °C without shaking for 15 min to facilitate phage adsorption. The unadsorbed phage particles were removed by centrifugation at 4000 g for 5 min. The pellet was resuspended in 5 ml of LB containing 20 mM sodium citrate to prevent further propagation of phage. The cell suspension was then incubated at 37 °C for 30 min without shaking for the phenotypic expression of the antibiotic resistance. The mixture was then centrifuged and the pellet was resuspended in 0.3 ml of citrate buffer (see Section 2.6 for composition). 100 µl aliquots were plated on tetracycline plates, supplemented with 2.5 mM sodium citrate. The transduced JM101 was named as MJMRH.

2.14.4 Generation of \( rpoS \) disruption mutant

The \( rpoS \) disruption mutant was generated by homologous recombination. A 753 bp internal fragment of \( rpoS \) gene was amplified in PCR using RPOSKOF, containing EcoRI site, as forward primer, and RPOSKOR, containing HindIII site, as reverse primer (Table 2.1). The amplified product was digested with EcoRI and HindIII and ligated to suicide vector pME3088. The recombinant plasmid thus generated was named as pMERPOS'. S17-1 cells were transformed with pMERPOS'. It was transferred to Lz4W in a biparental mating between Lz4W and S17-1 carrying pMERPOS'. Because it contains an internal fragment of \( rpoS \), a single recombination event would disrupt \( rpoS \) reading frame. For the selection of exconjugants we took the advantage of Lz4W being naturally ampicillin resistant. The exconjugants were selected on ampicillin and tetracycline plates. For a schematic diagram of the whole procedure, see Figure 6.2.
2.15 Molecular techniques

2.15.1 Agarose gel electrophoresis

Routine checking of the DNA samples were carried out using 0.8–1% small (5 cm long) agarose gel, cast in either 0.5 x TAE or TBE with ethidium bromide. Ethidium bromide was added at final concentration of 0.5 µg/ml either directly into molten agarose or in the tank buffer after the electrophoresis. DNA samples were loaded in DNA loading dye containing 30% glycerol and 0.25% bromophenol blue. The electrophoresis was carried out in 0.5 x TAE or TBE. For separation of genomic DNA digest longer gel (15–20 cm long) were used. For genomic DNA the electrophoresis was carried out at constant voltage of 1–2 V/cm for 12–16 h.

2.15.2 Genomic DNA purification

Genomic DNA from bacterial cultures were purified by a modified protocol originally presented in Towner (1991). 100 ml overnight grown bacterial culture was spun down and washed with 40 ml of TE and resuspended in 3.2 ml of lysis buffer containing 50 mM Tris-Cl (pH 8.0) and 0.7 M sucrose. Then the following solutions were added to the resuspended culture—0.6 ml lysozyme (20 mg/ml), 0.6 ml of 0.5 M EDTA (pH 8.0), 0.5 ml of 10% SDS and 5 µl RNase (100 mg/ml). The whole mixture was incubated for 10 min at room temperature. Then, 250 µl of Proteinase K (10 mg/ml) was added to the mixture, and incubated at 50°C for 30 min. The mixture was then extracted twice with phenol:choloroform (1:1 ratio) and the DNA precipitated by adding 1/10th volume of 3 M sodium acetate and 2.5 volume of ethanol. The DNA was spooled by a glass rod and dipped several times in 70% ethanol before dissolving in sterile water.

The purity of the extracted DNA was then checked by standard spectrophotometric method by measuring OD$_{260}$ and OD$_{280}$ of the solution. The quantity was also measured by running an aliquot in agarose gel and comparing with the known standard.

The whole process was scaled down for extraction of DNA from 3 ml culture.

2.15.3 Plasmid DNA purification

Depending on the amount or the quality of plasmid DNA, various methods were used. Small-scale (mini prep, from 3 ml culture) preparations were routinely done using alkaline lysis method described below. For higher quality of plasmid DNA, commercial kits were used. Plasmid DNA from large cultures
were prepared first by alkaline lysis followed by CsCl density gradient centrifuga-

tion.

2.15.3.1 Alkaline lysis

Small-scale preparation of plasmid DNA was made by the alkaline lysis method
(Birnboin and Doly 1979) as described in Sambrook et al. (1989) with some
modifications. The first solution for bacterial resuspension used, was TE (con-
taining 100 μg/ml RNase) instead of the usual (50 mM glucose; 25 mM Tris-Cl
and 10 mM EDTA).

3 ml bacterial culture bearing the plasmid was centrifuged. Bacterial pel-
let obtained was resuspended in 100 μl of TE (containing 100 μg/ml RNase).
200 μl of Solution 2 (0.2 N NaOH, 1% SDS) was added and the contents of the
tube were mixed by inverting the tube several times. This was followed by the
addition of 150 μl of ice-cold Solution 3 (Made by adding glacial acetic acid
to 5 M potassium acetate till pH becomes 4.8. For 100 ml of solution—60 ml
of 5 M potassium acetate, 11.5 ml of glacial acetic acid and 28.5 ml of water.)
and gentle mixing. The tube was incubated on ice for 5 min and centrifuged
at 12,000 g for 10 min at 4 °C. The supernatant was extracted with an equal
volume of phenol:chloroform and the DNA was precipitated with two volumes
of absolute ethanol followed by 70% ethanol rinse. The plasmid DNA was then
checked on a 0.8% agarose gel and stored at -20 °C.

This DNA was suitable for routine procedures such as restriction diges-
tion, preparation of radiolabelled probe and manual sequencing.

2.15.3.2 Large-scale isolation of plasmid

Large-scale preparation (from 500 ml culture) of plasmid DNA was carried out
by CsCl density gradient centrifugation as described in Sambrook et al. (1989)
with some modifications. The major difference was at the centrifugation step
as described below.

Bacterial culture (500 ml) was harvested and resuspended in 8 ml of Solu-
tion 1 (50 mM glucose; 25 mM Tris, pH 8.0 and 10 mM EDTA). To the suspen-
sion 800 μl of lysozyme (10 mg/ml) was added. Then, 16 ml of Solution 2 (0.2 N
NaOH and 1% SDS) was added to the suspension and mixed thoroughly. The
content of the tube was mixed gently by inverting the tube. 12 ml of Solution 3
(see Section 2.6) then added to the tube and centrifuged at 12,000 g for 15 min.
Supernatant was collected and the plasmid DNA was precipitated by adding 0.6
volume of isopropanol. The DNA pellet was rinsed with 70% (v/v) ethanol and
air-dried.

For CsCl density gradient centrifugation the DNA was resuspended in ex-
actly 4 ml of TE. To this solution, exactly 4.5 g solid CsCl (Serva) was added
followed by 0.5 ml of ethidium bromide (10 mg/ml). The content of the tube was mixed and centrifuged at 12,000 g for 15 min at room temperature. The clear solution from the top of the tube was loaded in Beckman Quick-seal tubes, sealed and centrifuged at 72,000 rpm for 5.5 h in a VTI80 rotor in Beckman L8-80 ultra-centrifuge.

After centrifugation, the supercoiled plasmid band was collected by piercing the tube with hypodermic syringe. Ethidium bromide was removed from the DNA by extracting several times with 1-butanol, and the DNA solution was dialized against two liters of TE for 24 h. After dialysis the DNA was precipitated with standard ethanol precipitation method.

The DNA isolated by procedure gave the best possible quality plasmid DNA.

2.15.3.3 Purification by commercial kits
Plasmids were prepared from 3 ml and 100 ml cultures by Wizard™ Mini and MidiPrep systems from Promega Corporation, USA, according to manufacturer’s protocol. DNA prepared through these commercial kits were mainly used for automated DNA sequencing.

2.15.4 Restriction digestion
All the restriction digestion were performed according to the protocol supplied by the manufacturer, typically, in a reaction volume of 20–50 μl.

2.15.5 Dephosphorylation of vector
When cut with single enzyme or when blunt ended, the vector was dephosphorylated using shrimp alkaline phosphatase (SAP) before ligation. Digested vector DNA (~50 ng) was dissolved in 7 μl water and to this 1 μl dephosphorylation buffer (10 X, supplied) and 1 μl SAP (1 unit) was added. The mixture was incubated for 10 min at 37 °C for staggered-ended vector, and 60 min at 37 °C for blunt-ended vector. The SAP was inactivated for 15 min at 65 °C. The dephosphorylated vector was used directly for ligation.

2.15.6 Ligation
Typically, 50–100 ng of DNA was used in each ligation reaction in 10–20 μl reaction volume with 0.05 Weiss units of T4 DNA ligase. The vector to insert ratio was maintained at 1:1 or 1:3. The reaction was carried out routinely at 16 °C for 12–16 h.
2.15.7 Extraction of DNA from agarose gel

DNA fragments were purified from agarose gel by GENE CLEAN™ (BIO 101) or QiaQuick™ (QIAGEN) gel extraction kit according to manufacturer's protocol.

2.15.8 Making of partial genomic library

Partial genomic library of Lz4W was made in plasmids to clone various genes of interest. In this method, genomic DNA was digested with several restriction enzymes and the digested DNA was analyzed by Southern hybridization (Section 2.15.12) with a suitable probe to find out the size of the restriction fragment that hybridizes to the probe. The enzyme that produced clonable fragment (up to 6 kb) was chosen to make the library. If no enzyme gave the proper size band the DNA was digested with two enzymes to bring the size of the band down to clonable range. The size of the fragment was noted very carefully.

Large quantity (~100 μg) of genomic DNA was then digested and separated in 0.8% TAE agarose gel in several lanes. A suitable molecular weight marker was loaded in one of the lanes. The gel was cast without ethidium bromide and was run in constant voltage (2–3 V/cm) overnight. After the run, the molecular weight marker lane was cut off from the gel and stained separately by soaking in ethidium bromide containing tank buffer and the marker band positions were marked on a transparent polythene sheet. The sheet was then placed on top of the rest of the gel, and around 1 cm thick slices were cut off from the required region of the gel using the molecular weight as guide. DNA was then isolated from these slices as described in Section 2.15.7. A small aliquot from each of these size fractionated DNA samples was run in agarose gel and hybridized with the same probe in Southern hybridization to check for the presence of the required DNA fragment.

Approximately, 50 ng of DNA was then ligated with 50 ng of pBluescriptII KS+ vector, cut with suitable restriction enzyme (for vector cut with single enzyme, it was dephosphorylated as described in Section 2.15.5). Around 1–2 μl of the ligation mix was electroporated into E. coli DH10B cells and plated on LB plates with ampicillin. The positive clone was then detected using colony blot, described in Section 2.15.13.

2.15.9 Cosmid genomic library

A cosmid genomic library from Lz4W was made in cosmid pLAFR3 (Staskawicz et al. 1987). The overall protocol followed was as described in Ausubel et al. (1991). The enzyme PstI was chosen for making the library for the random distribution of its recognition sites in Lz4W genome. The cosmid pLAFR3 was
the cosmid of choice because of the presence of a unique PstI site in its MCS and its broad host-range replicon. The various steps of making the cosmid library is described below.

2.15.9.1 Standardization of partial digestion

The partial digestion of the genomic DNA was standardized by incubating a fixed amount of genomic DNA with various units of PstI (2-0.007 units). Briefly, 20 μg of genomic DNA in 22 μl volume was mixed with 22 μl of 10× restriction enzyme buffer and 176 μl of water. From the mixture, 20 μl aliquot each, was distributed in 11 tubes. PstI (8 units) was added in the first tube and the content was mixed by pipetting. From the content, 20 μl was transferred to the second tube and the serial dilution was carried out to tenth tube, with each time 20 μl content was transferred to the next. The first tube was discarded and the eleventh tube was kept aside as negative control (minus enzyme). All the ten tubes were incubated at 37 °C for one hour. The samples were run in 0.3% agarose gel with a 1% agarose base, with a very low constant voltage (3 V/cm) for 6 h. The concentration of the enzyme that produced the maximum product at around 20 kbp region was chosen for the next step.

2.15.9.2 Large-scale partial digestion of DNA

For large-scale partial digestion about 200 μg of genomic DNA was taken in 400 μl of 1× restriction enzyme buffer. Digestion was carried out with 30 units of PstI at 37 °C for 1 h. An aliquot of the sample was checked for digestion by running in 0.3% agarose gel. The same reaction was performed in batches of four.

2.15.9.3 Size fractionation of restriction fragments

Four 12 ml continuous gradient of NaCl (5-25%) were prepared in Beckman SW41 tubes using a commercial gradient maker. 200 μg of partially digested DNA was loaded onto each gradient and centrifuged at 37,000 rpm for 4.5 h at 4 °C in Beckman SW41 rotor. Fifty fractions, each of 250 μl, were collected from each tube. The fractions were analyzed on 0.3% agarose gel. The fractions of size 20-30 kb were pulled together and precipitated with ethanol.

2.15.9.4 Preparation of cosmid DNA and ligation

About 30 μg of cosmid DNA (pLAFR3) was digested with 40 units of PstI in a reaction volume of 150 μl. The digestion was carried out at 37 °C, overnight. The completion of the digestion was checked by analyzing an aliquot in 0.8% agarose gel.
The linearized cosmid was dephosphorylated by adding 2 μl (10 units/μl) of calf intestinal phosphatase (CIP) directly into the digestion tube and incubating the DNA at 37 °C for 30 min. After the reaction, the digested and dephosphorylated cosmid was purified by phenol:chloform extraction and ethanol precipitation.

For ligation, 9 μg of insert and 3 μg of vector were precipitated together by ethanol, and resuspended in 10.5 μl of 1× ligation buffer. The ligation was carried out at 15 °C, overnight in 15 μl reaction volume, in presence of 4 units of T4 DNA ligase. The efficiency of ligation was checked by analyzing an aliquot on 0.5% agarose gel.

2.15.9.5 Packaging

Packaging of ligated cosmid and inserts were carried out reaction was done using Gigapack II (Stratagene) packaging extract as specified by supplier.

2.15.9.6 Titering the library

Culture (50 ml) of E. coli DH10B cells were grown in LB supplemented with 500 μl of 1 M MgSO4 and 500 μl of 20% maltose. The cells were harvested and resuspended in 12.5 ml of 10 mM MgSO4 and stored at 4 °C until further use. Just before use the cells were diluted to OD600 of 0.5 in 10 mM MgSO4. Three dilutions of the packaged DNA (1:10, 1:30, 1:50) were prepared with SM buffer (see Section 2.6 for composition). 25 μl of each dilution was mixed with 25 μl of diluted DH10B cells and incubated at room temperature for 30 min. About 200 μl of LB broth was added to each sample and incubated at 37 °C for 1 h. After the incubation the cells were harvested by centrifugation for 1 min, and resuspended in 800 μl of fresh LB. Aliquot of 200 μl each, of the resuspended cells was plated on four plates. The dilution of 1:30 was found to be suitable for amplification.

2.15.9.7 Amplification and storage of the library

The packaged phage particles (20 μl) were diluted to 600 μl in SM buffer and incubated with 600 μl of E. coli DH10B cells (OD600 ~0.5) and incubated at room temperature for 30 min. Four volumes of LB (4.8 ml) was added to the cells and incubated 1 h at 37 °C with shaking. The cell suspension was distributed in four microfuge tubes and cells were pelleted down by quick spin and resuspended into 1 ml of LB for each tubes. The cells were spread on twenty 153 mm LB agar plates supplemented with tetracycline, X-gal and IPTG.

White colonies were picked up from each plates and individual clones were inoculated into 100–150 μl of LB containing tetracycline in 96-well microtiter
plates. The plates were incubated overnight at 37°C. Equal volume of 40% glycerol was added to each well and the plates were stored at −70°C. Around 2,200 individual clones were stored frozen by this method.

2.15.9.8 Screening of the library

A custom-made inoculation devise, with 96 projections was used to make replica of the 96-well microtiter plates on Hybond™N+ nylon membrane. Each membrane were treated and probed as discussed in Section 2.15.13.

2.15.10 Transformation

Depending on the transformation efficiency required, various transformation protocols were used for *E. coli*. *P. syringae* cells were transformed only by electroporation.

2.15.10.1 Calcium chloride method

For routine plasmid transformations, where efficiency of transformation were not important, the "classical" Mandel and Higa (1970) calcium-chloride method were used. The following protocol was a modification of the protocol described in Brown (1991).

An overnight grown culture of the *E. coli* was diluted 100 times in fresh LB medium (100 μl cells inoculated in 10 ml) and subcultered till OD$_{600}$ reached 0.4–0.5. The culture was chilled on ice for 15 min. All the steps thereafter were carried out at 4°C. The cells were harvested and resuspended in \( \frac{1}{2} \) volume of the original culture of sterile ice-cold 100 mM CaCl$_2$. The cells were again harvested and resuspended in \( \frac{1}{10} \) volume of ice-cold 100 mM CaCl$_2$. This suspension was incubated on ice for at least 1 h. To 100 μl of this suspension, DNA (10–100 ng of DNA in less than 10 μl volume) was added. The mixture was incubated on ice for another 30 min and then transferred to a 42°C water bath for exactly 90 s. Immediately, 0.9 ml of LB medium was added to the tube and the tube was incubated at 37°C for 30–45 min for phenotypic expression of the antibiotic marker before plating them on selective media at various dilutions.

2.15.10.2 Rubidium chloride method

Most of the routine cloning experiments were carried out with competent cells made according to the method described by Hanahan (1985) with a few modifications. In this protocol a single colony of *E. coli* cells was inoculated into 3 ml of LB medium and incubated at 37°C overnight. Aliquot of 500 μl of this overnight culture was inoculated into 50 ml of SOB medium and incubated at 37°C at 200 rpm till the OD$_{600}$ reached ~0.5. The culture was then chilled
on ice for 15 min and the cells were harvested by centrifugation at 4000 rpm for 15 min at 4°C. The supernatant was completely drained and the pellet was gently resuspended in 20 ml (0.4 volume) of ice-cold RF1 buffer (see Section 2.6) and incubated on ice for 20 min. The cells were then harvested and resuspended in 2 ml (0.04 volume) of the ice-cold RF2 buffer (Section 2.6) and incubated on ice for 15 min. The cells were then distributed into prechilled microfuge tubes in 40 μl aliquots; flash frozen in liquid nitrogen and stored at −70°C. Whenever required, one aliquot of cells was thawed on ice and mixed with DNA (50–100 ng) and incubated for 30 min on ice. After incubation the tube was transferred to 42°C water bath for 90 s and 1 ml of LB or SOB or SOC added to the tube and incubated at 37°C for 30–60 min and plated on selection plate.

The competent cells prepared by this method generally yielded a transformation efficiency of $5 \times 10^6$ to $1 \times 10^7$ colonies/μg of pUC19 DNA, and were stable up to three months at −70°C.

2.15.10.3 Ultracompetent cells

Ultracompetent cells were used for cloning experiments requiring high efficiency transformation. The cells were prepared according to the method described in Inoue *et al.* (1990). A single colony of *E. coli* recipient cell was inoculated into 3 ml of LB and incubated at 37°C overnight. Aliquot of 2.5 ml of this overnight culture was inoculated into 250 ml of SOB medium and incubated at 18°C at 200 rpm, till the OD$_{600}$ reached 0.6–0.7. The culture was chilled on ice and the cells were harvested by centrifugation at 4000 rpm for 15 min at 4°C. The cells were then resuspended in 80 ml of ice-cold transformation buffer (TB, see Section 2.6) and incubated on ice for 10 min. The cells were harvested and resuspended in 20 ml of TB containing 7% DMSO. The cells were then distributed in 100 μl aliquots; flash frozen in liquid nitrogen and stored at −70°C. The transformation were done by heat-shock as in case of rubidium chloride method discussed in Section 2.15.10.2.

Ultracompetent cells prepared by this method yielded a transformation efficiency of $1 \times 10^8$ colonies/μg of pUC19 DNA and were stable for six months at −70°C.

2.15.10.4 Electroporation

For making plasmid genomic library or when very high efficiency of transformation was required the *E. coli* cells were transformed by electroporation.

The recipient cells were made electrocompetent by the following method. Cells were grown in large volume (one liter) till the OD$_{600}$ reaches 0.5–0.6. Culture was then cooled and washed three to five times with sterile ice-cold water
(the total washing should be at least the same volume of the original culture). The last washing was carried out in sterile ice-cold 10% glycerol. The cells were then resuspended in 500 μl of sterile ice-cold 10% glycerol, snap frozen in 40 μl aliquots in liquid nitrogen, and stored in -70°C. The electrocompetent cells thus prepared were transformed by electroporation using Bio-Rad Gene Pulser™ II, according to manufacturer's protocol.

2.15.11 Preparation of radio-labelled probes

Oligonucleotides were radio-labelled at the 5'-end with γ-[32P]dATP using T4 polynucleotide kinase (PNK). About 10 picomoles of primer was incubated at 37°C for 30 min with 40 μCi of γ-[32P]dATP and 5 units of PNK in 50 μl reaction volume.

Longer double-stranded DNAs were radio-labelled by random-priming with α-[32P]dATP using Klenow enzyme and random hexamer oligonucleotides by a commercial random-priming kit (BARC, India), according to manufacturer's protocol. About 50 ng of DNA was labelled in each reaction.

After the reaction, the probe was purified from the unincorporated nucleotides by Sephadex G25 or G50 spin-column chromatography. Sephadex G25 was used for oligonucleotide probes and G50 was used for probes longer than 150 bp. Briefly, a 1 ml disposable syringe was plugged with siliconized glass-wool (Supelco) and filled with Sephadex G25/50 slurry previously equilibrated with TE (pH 8.0). The column was packed by centrifugation at 2,500 rpm for 5 min in a Sorvall HB-4 swing-out rotor. Radio-labelled probe (100 μl) was then loaded on the column and the probe was purified by centrifugation, again at 3,000 rpm for 5 min.

2.15.12 Southern hybridization

Southern hybridization was performed as described in Parry and Aalhey (1995). Briefly, DNA samples were electrophoresed in appropriate agarose gels. After electrophoresis, the DNA was transferred onto positively charged nylon membrane by capillary transfer overnight in 0.4 N NaOH, a method originally described in Reed and Mann (1985). After transfer, the membrane was rinsed with 2 × SSC air-dried and stored at room temperature until further use.

Hybridization was carried out in buffer described in Section 2.6 (Church and Gilbert 1984) at 65°C, overnight for double-stranded probes and 55°C overnight for oligonucleotide probes in sealed polyethylene bag. After the hybridization, the membrane was washed to remove the unbound probe. In the case of oligonucleotide probe, the membrane was washed with 6 × SSC thrice at room temperature with each wash lasting for 20 min, and in the case of longer probes the membrane was washed with 0.5–0.1 × SSC with 0.1% SDS at 68°C.
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for appropriate times and then exposed to phosphorimager (Fuji) screen or X-ray film.

2.15.13 Colony blot

Genomic library of Lz4W in plasmid or cosmid, and transformed cells were screened for positives using colony blotting. In the case of genomic library the colonies were either grown or spread on Hybond™-N+ membrane placed on agar surface. In some cases already grown colonies were lifted by placing the membrane on top of the colonies on agar surface. After colonies were transferred on the membrane, it was placed, colony side up, on a pad of Whatman 3M filter paper soaked in denaturing solution (1.5 M NaCl; 0.5 M NaOH) for 7 min. The membrane was then placed on another pad of filter paper soaked in neutralizing solution (1.5 M NaCl; 0.5 M Tris-HCl pH 7.2; 0.001 M EDTA) for 3 min. The last step was repeated again by changing the filter paper. The membrane was then washed vigorously in 2 × SSC and wiped gently with a cotton ball soaked in 2 × SSC to remove cell debris. The membrane was then transferred on a dry filter paper and air-dried. The DNA was fixed on membrane using alkali fixation procedure (Reed and Mann 1985) by placing it on the surface of a filter paper soaked in 0.4 M NaOH for 20 min. The membrane was then rinsed with 2 × SSC for 1 min, air-dried and stored at room temperatures until further use. The hybridization was carried out using conditions described in Section 2.15.12.

2.15.14 Polymerase Chain Reaction (PCR)

For PCR using genomic DNA as a template about 250 ng DNA was used as template and for plasmid DNA 10–20 ng DNA was used as template in 50 or 100 μl reaction volume. Reactions were performed with 200 μM of dNTPs, 1 picomoles/μl each of forward and reverse primers and 1.5 units of Taq DNA polymerase.

For genomic PCR, the cycle conditions were as follows: initial 3 min denaturation at 94 °C. Three cycles of denaturation at 94 °C for 1 min, annealing at 37 °C for 1 min, and extension at 72 °C for 2 min. Followed by 25 cycles of denaturation at 94 °C for 1 min, annealing at 50 °C for 1 min, and extension at 72 °C for 2 min. The reaction was terminated with 5 min extension at 72 °C.

For plasmid DNA template, the cycle conditions were as follows: initial 5 min denaturation at 95 °C. Then 30 cycles with denaturation at 95 °C for 1 min, annealing at 50–55 °C for 1 min and extension at 72 °C for 1–2 min. The reaction was terminated with extension at 72 °C for 5 min.

The PCR reactions were performed in either Perkin Elmer or MJ Research's DNA Engine™ thermal cycler.
2.15.15 DNA sequencing

The DNA sequencing reaction was carried out on double stranded plasmid template using dye terminator chemistry using Big Dye Terminator sequencing kit (Applied Biosystems, USA) and the sequences determined using either ABI Prism 3700 or ABI prism 377 automated DNA sequencer (Applied Biosystems, USA).

2.15.16 SDS-PAGE

Whole cell proteins were separated on 10–12% SDS-PAGE according to the classical protocol devised by Laemmlı (1970) as described in Sambrook et al. (1989).

For separating whole cell proteins, cells were harvested from various growth phases and resuspended at calculated OD of 20 in 1 × sample buffer (see Section 2.6 for composition). This cell suspension was boiled for 5 min and debris was removed by centrifuging at 12,000 g for 15 min. Clean supernatant in a volume of 25–50 μl was loaded in each lane.

For analysis of cell extract, measured volume of crude extracts containing equal amounts of protein were taken in 20 μl of water. Five microliters of 5 × sample buffer was added to each tube. The samples were boiled for 5 min and equal amount loaded in each lane.

Gels were run at constant current of 20 mA for stacking gel, and 40 mA for resolving gel. After the run, if the gel was for western blotting, it was processed as described in Section 2.15.17; otherwise, the gel was stained in Coomassie Brilliant Blue (0.25 g in 30% methanol and 10% acetic acid) for minimum 1 h and destained in 30% methanol and 10% acetic acid mixture.

2.15.17 Immunoblotting with anti-RpoS antibody

RpoS protein level was detected using a mouse anti-RpoS polyclonal serum generated against P. putida RpoS (Ojangu et al. 2000; kind gift from M. Kivisaar). Polyclonal anti-sera generated against E. coli RpoS (kind gift of R. Hengge-Aronis) did not give consistent result. For comparative quantitation of RpoS, protein samples were prepared by either sonication in phosphate buffer, as described in Section 2.10 or by direct cell lysis in SDS-PAGE sample buffer. The equalization of the protein amount in the samples were done by either measured protein concentration or by resuspending the cell pellet to an estimated final OD of 20 in 1× SDS-PAGE sample buffer. Typically 20–40 μl of samples were loaded in each lane.

After separation on SDS-PAGE (see Section 2.15.16), the samples were transferred on to Hybond™ PVDF (polyvinylidene difluoride, Amersham) or ni-
trocellulose (Hybond™ C, Amersham) using BIO-RAD Trans-Blot™ semi-dry transfer apparatus, in transfer buffer, described in Section 2.6. The transfer was carried out for 1.5 h with current set at 0.8 mA/cm². After transfer, the efficiency was checked by staining the blot in 0.5% Ponceau S (Sigma) in 1% acetic acid. The excess stain was removed by rinsing the blot several times with water and the positions of molecular weight marker proteins were marked on the blot. If required, the blot was then scanned using a flat-bed scanner for reference.

Next, the blot was blocked in 5% not-fat milk in TBS-T (see Section 2.6) for 2–12 h. The blot was then washed three times with TBS-T and incubated with primary antibody (1:250 dilution, in TBS-T with 1% BSA) for 1.5–2 h. After incubation, the blot was washed thrice with TBS-T, each wash for 15 min. The blot was then incubated in anti-mouse IgG secondary antibody (in TBS-T with 1% BSA, with 1:10,000 dilution) for 1 h. The blot was subsequently washed three times with TBS-T to get rid of excess antibody. After rinsing with water, the blot was incubated in 10 ml of AP buffer (see Section 2.6) in presence of 66 μl of NBT (5% solution in DMF) and 33 μl of BCIP (disodium salt; 5% aqueous solution) till the signal appeared.