Materials and Methods

MI. Materials

Chemicals and Biochemicals:

Fine chemicals were purchased from Sigma Chemical Co., St. louis, USA; other chemicals like K_2SO_4 ; Glucose; Sucrose, Maltose were purchased from Qualigens, India. Sodium dodecyl sulphate was from S.D. fine chem. Ltd, India. Chemicals used for TLC like silica gel G and silica for column, Triflouroaceticacid (TFA), solvents like acetonitrile, chloroform, ethyl acetate, methanol, HPLC grade H_2O was from either Merk Research labroratories, USA or E.Merk (India) Ltd., Mumbai, India. Agarose, Methanol, isopropanol, proline, lysozyme were obtained from SRL India. Culture media including Luria broth, Luria agar, peptone, tryptone, yeast extract, bactopeptone; tryptone Soya broth; Malt extract; cas-amino acids; agar agar were obtained from Hi-media, India.
Chemicals for photography were purchased from Kodak India Ltd., Mumbai, India.
All other chemicals used in this study were also of analytical grade and obtained from local sources.

Antibiotics: Ampicillin; Kanamycin; Apramycin and Chloramphenicol were obtained from Sigma Chemical Co. U.S.A. Tetracycline, Nalidixic acid, Trimethoprim, Carbinicillin, Erythromycin was obtained from local market or Hi-media, India and was of analytical grade.

Enzymes And DNA Labelling Kit : DIG- labeling kit, Restriction enzymes and T4 DNA Ligase were purchased from Promega, Madison, USA; Roche Molecular Biochemicals, Mannheim, GDR; MBI Fermentas, Vinuis, Lithuania. RNase was purchased from Sigma chemical Co., St. louis, USA.

Bacterial Strains: The list of bacterial strains, plasmids and phage used in this study are given in table M1.

Table No. M .1. Bacterial strains, Plasmid and Lambda.

Strains	Genotype / phenotype	Source/References
***Streptomyces* strains**		
S.flavisclerotícus	PKS^+ Produces yellow colored diffusible pigment	MTCC
JP2	*S. falvisckeroticus pks*⁻ derivative constructed by deleting 12 kb of PKS DNA by genetic recombination	present study
***E. coli* Strains**		
E.coli (DH5α)	*deoR, endAl, gyrA96, hsdR17 (rk⁻ mk⁺), recAl, supE44, thi-1, Δ (lac ZyA - arg F),* U169, Φ80*dlacZ*ΔM15.	Lab Collection
E.coli(MC 1061)	*araD739, Δ(ara⁻ leu)7696, Δ(lac)174galU, galK, hsdR2 (rk⁻mk⁺), mc Bl, rpsl(str)*	Lab Collection
E.coli (S17.1)	*recA⁻, mob⁺ , thi, pro, hsdR⁻, hsdM⁺*/RP4-2-Tc::Mu-Km::Tn7	MMBL Lab Collection
Micrococcus luteus	produces shiny yellow colored colony ATCC4698	Lab collection
Plasmids		
pBluescriptII	Ap^r	Lab collection
pBR322	Ap^r	Lab collection
pKC505	Am^r	Lab collection
pGM160	Gm^r, Tsr^r, Ap^r	Lab collection
pSET152	Am^r	Lab collection

Growth media: Media, solutions for media, buffers, solutions for plasmid preparation, southern hybridization and other miscellaneous solutions, glasswares and plasticwares were sterilized by autoclaving at 15 lbs/ in^2 at 121^0C for 20min, unless otherwise specified.

MII. Culture Media for *Streptomyces*:

MII.1 Solid media:

- **R_2YE medium** (Thompson *et. al*, 1980)

Sucrose	-	10.3 gm
K_2SO_4	-	0.25 gm
Glucose	-	10 gm
Difco casamino acids	-	0.1 gm
Agar	-	2 %

Make up the final volume to 1000 ml with D/W. After autoclaving, at time of use, melt the medium to add following sterile solutions

For 100 ml		
KH_2PO_4 (0.5 %)	-	1.0 ml
$MgCl_2$ (2 M)	-	2.5 ml
$CaCl_2$ (5 M)	-	0.4 ml
TES buffer (5.73 %, pH 7.2)	-	0.4 ml
Trace element solution	-	0.2 ml
Difco yeast extract (10 %)	-	5.0 ml
L – Proline (20 %)	-	1.5 ml
NaOH (10 N)	-	0.03 ml (sterilization not need}

Composition of Trace element solution (for 1000ml) :

$ZnCl_2$	-	40 mg
$FeCl_3 . 6H_2O$	-	200 mg
$Cu Cl_2. 2H_2O$	-	10 mg
$MnCl_2. 4H_2O$	-	10 mg
$Na_2 B_4O_7 . 10H_2O$	-	10 mg
$(NH4)_6 MO_7 O_{24} . 4H_2O$	-	10 mg

- **R2 medium** (Okanishi et al, 1974 ; Hopwood Wright 1978)

  Same as R2YE only Difco yeast extract is to be excluded.

- **MBA medium**

Peptone	-	0.2 g
Difco yeast extract	-	0.1 g
NaCl	-	0.1 g
Beef extract	-	0.1 g
Glycerol	-	1.0 ml
pH	-	7.2
Agar	-	2%

  (For soft MBA add only 1% agar)

  Make up the final volume up to 100 ml with D/W

- **Soyabeam Mannitol Medium (SM)**

Soybean Meal	-	2.0 %
Mannitol	-	2.0 %
pH	-	7.2
Agar	-	2%

- **YS**

Yeast extract	-	0.3 %
Soluble starch	-	1.0 %
pH	-	7.5
Agar	-	1.5 %

  D/W to make final volume to 100 ml

- **S Media**

$MgCl_2$	-	0.5mM
KH2PO4	-	1.0mM
Nitrate	-	10mM
Glucose	-	125mM
Microelements	-	0.2%

- **Nitrate defined yeast extract medium (NDYE)**

$NaNO_3$	-	0.850 g
K_2HPO_4	-	0.174 g
$MgSO_4.\ 7H_2O$	-	0.123 g
HEPES	-	4.775 g
Trace elements	-	2.0 ml
*Maltose	-	5 %
Yeast extract	-	0.5 %
pH	-	7.2

D/W to make the volume to 750 ml

*To be autoclaved separately and added at the time of inoculation.

- **Soft Nutrient Agar (SNA)**

For 1000ml:

Difco Nutrient broth powder	-	8 g
Agar	-	0.75 %
pH	-	7.2

MII.2. Liquid Media:

- **Yeast extract- malt extract medium (YEME)**

  For 1000ml:

yeast extract	-	3 g
Bacto – Peptone	-	5 g
malt extract	-	3 g
Glucose	-	10 g
Sucrose	-	340 g
pH	-	7.2

  After autoclaving, add: $MgCl_2 . 6H_2O$ (2.5M) 2 ml / liter

  YEME (10% Sucrose)

  YEME (20% Sucrose)

- **Nitrate Defined Media (NDM)**

  For 1000ml

NaNo3	-	0.850g
K2HPO4	-	0.174g
MgSO4.7H2O	-	0.123g
HEPES	-	4.775g
Trace element	-	2.0ml
Maltose	-	5%
Distilled water	-	750ml
Maltose (after autoclaving)	-	250ml
PH	-	7.2

- **Tryptone Soya Broth (TSB)**

  For 1000ml:

Tryptone Soya broth powder	-	30 g
Distill water		1000 ml

- **½ X TSB + ½ X YEME**

  In 1:1 ratio (pH7.2)

  (Add $MgCl_2$ after autoclaving at 10 mM Concentration)

- **2X YT Medium**

  For 1000ml:

  To 900 ml of deionized H_2O, add:

Tryptone	-	16 g
Yeast extract	-	10 g
NaCl	-	5 g

  pH adjust to 7.0 with 5N NaOH

MIII. Media used for culturing *E.coli*:

MIII.1. Solid Media:

- **Luria Agar (LA)**

Agar Agar	-	10 g
Bacto tryptone	-	5 g
yeast extract	-	5 g
NaCl	-	5 g
Glucose	-	1 g
Distilled water	-	1000 ml
pH	-	7.2

- **Luria Agar (LA) from Hi-media.**

  LA ready powder - 35 gm/ 1000ml distilled water.

MIII. 2. Liquid media:

- **Luria Broth (LB)** - same as LA without agar-agar

- **Nutrient Broth (NB)**

  Nutrient broth powder - 8 g / 1000ml distilled water

- **Nutrient Broth (NB)**

- **Terrific broth (TB)**

Tryptone	-	12g
Yeast extract	-	24g
Glycerol	-	4ml
D/w	-	900ml

*** phosphate buffer**

KH_2PO_4	-	2.31g (0.17M)
K_2HPO_4	-	12.54 (0.72M)
D/W	-	100ml

* Before inoculation, add 100ml phosphate buffer to 900ml of TB.

MIV. Reagents for plasmid preparation

- **Alkaline lysis solution I (GTE)**

  50 mM glucose

  25 mM Tris – Cl (pH 8.0)

  10 mM EDTA (pH 8.0)

  Prepare solution I from standard stocks of 2 M Glucose, 1M Tris – Cl (pH 8.0) and 0.8 M EDTA (pH 8.0) and make up the final volume with autoclaved D/W. (No need to autoclave the final solution).

- **Alkaline lysis solution II**

  0.2 N NaOH (freshly diluted from a 10 N stock)

  1 % (w/v) SDS

  Prepare solution II fresh and use at RT.

- **Alkaline lysis solution III**

5 M potassium acetate	60.0 ml
Glacial acetic acid	11.5 ml

H_2O 28.5 ml

The resulting solution is 3 M with respect to potassium 5 M with respect to acetate.

Note: To be use cold.

- **Buffered phenol**

  Phenol obtained commercially was distilled at 160^{0}C and stored at 4^{0}C in aliquots. To make buffered phenol, distilled phenol was equilibrated first with equal volume of 1M Tris-HCl (pH 8) and then equal volume of 0.1M Tris-HCl (pH 8). 8-hydroxyquinoline was added to a final concentration of 0.1% and stored at 4^{0}C.

- **Phenol : chloroform Isoamyl-alcohol.**

  Ratio used - 1:1

- **Chloroform: Isoamyl –alcohol mixture**

  Ratio used - 24:1

- **10 X Tris EDTA (TE)** pH 8.0

  100 mM Tris – cl (pH 8.0)

  10 mM EDTA (pH 8.0)

- **Sodium Acetate (3 M, pH 5.2)**

  Dissolve 408.3 g of sodium acetate.3H_2O in 800 ml of H_2O. Adjust the pH to 5.2 with glacial acetic acid. Adjust the volume to 1 liter with water.

MV. Buffers

- **P (protoplast) buffer** (Okanishi et al 1974; Hopwood and Wright, 1978)

  Make up the following basal solution:

Sucrose	-	103 g
H_2SO_4	-	0.25 g
$Mgcl_2$. $6H_2O$	-	2.0 g
Trace element solution	-	2 ml
Distilled water to	-	800 ml

  Dispense in 20 ml aliquots and autoclave

Before use, add to each tube the following sterile solutions

KH_2PO_4 (0.5 %)	-	0.40 ml
$Cacl_2$.$2H_2O$ (3.68 %)	-	0.16 ml
TES buffer (5.73 %, pH 7.2)	-	0.16 ml

- **T (Transformation) buffer** (Thompson et al 1982)

  Mix the following sterile solutions

Sucrose (10.3 %)	-	25 ml
Distilled water	-	75 ml
Trace element solution	-	0.2 ml
K_2SO_4 (2.5 %)	-	1 ml

  To 9.3 ml of the above solution add:

$Cacl_2$ (5M)	-	0.2 ml
Tris – maleic acid buffer	-	0.5 ml

  For use, add above solution and pre-sterilized PEG-1000 in ratio of 3:1 (v/w)

- **Tris – Maleic acid buffer**

  Make up a 1M solution of Tris and adjust to pH 8.0 by adding Maleic acid.

- **Restriction Enzymes and Ligase Buffer:**

  Buffers for restriction enzyme digestion and ligations were supplied by manufacturers and were used according to the instructions given.

MVI. Solutions for agarose gel electrophoresis

- **Running buffer: 50x TAE**

Tris-base	-	242g
Glacial acetic acid	-	57.1ml
0.5 M acetic acid (pH 8.0)	-	100ml
Distilled water	-	1000ml

  50x TAE was diluted to 1x prior to use.

Antibiotics Used

Table M.2: list of antibiotics used:

Antibiotic	Stock conc.	Working conc.
Ampicillin	100 mg / ml	50-100 μg / ml
Apramycin	100 mg / ml	50 μg / ml
Chloramphenicol	40 mg / ml	30 μg / ml
Kanamycin	100 mg / ml	50 μg / ml
Tetracycline	50 mg /ml	12-15 μg / ml
Nalidixic acid	30 mg / ml	30-40 μg / ml
Gentamycin	30mg/ml	15-20μg / ml

X-gal (2%)

X-gal - 20mg

Dimethyl sulphoxide - 1ml

IPTG (100mM)

IPTG - 23.8mg

D/W - 1ml

Solvent system for TLC

SS1 - $CHCl_3$: Methanol (9.3: 0.7 by volume)

SS2 - Ethyl acetate: Methanol (9:1 by volume)

Solvent system for HPLC

Solvent A – H20 + 0.1% TFA, Solvent B- ACN +0.1% TFA

MVII. Miscellaneous reagents:

DNA loading Dye (6X)

1. O.25% (w/v) Bromo phenol blue
   60% (w/v) sucrose
   100mM EDTA pH 8.

2. O.25% (w/v) Bromo phenol blue
   30% Glycerol

Ethidium bromide (10 mg/ml) - Add 1g of ethidium bromide to 100 ml of water. Stirr on a magnetic stirrer, for several hours to ensure that the dye has dissolved. Wrap the container in aluminum foil or transfer the solution to a dark bottle and store at RT.

Staining:	During electrophoresis	-	add 0.5-1 µg/ml agarose solution.
	After electrophoresis	-	add 0.5-2 µg/ml staining solution

RNase A – Dissolve RNase A at concentration of 10 mg / ml in 0.01 M sodium acetate (pH 5.2). Heat at 100°C for 15 minutes. Allow to cool slowly at RT. Adjust the pH by adding 0.1 volume of 1 M Tris-CL (pH 7.4). Dispense in aliquots and store at -20° C.

Sucrose solution (10.3 %)

0.1M CaCl2. $2H_2O$

0.1M $MgCl_2$

Sodium pyrophosphate (stock = 250 mm)

Sodium citrate

SDS (20 %)

Lysozyme (Sigma)

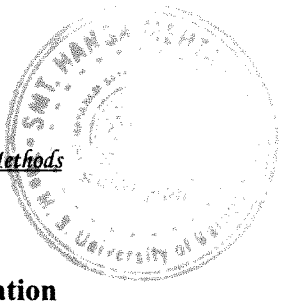
PEG (Sigma – 1000)

Silica gel G for TLC

Silica gel (200-400 mesh size) for column chromatography.

X-ray film Kodak X-ray film

X-ray film Devloper and Fixer : Ready to use from Kodak.



MVIII. Solutions for non radioactive Southern hybridization transfer:

- **Depurination buffer** (0.25 M HCl)

Conc. HCl	-	21 ml
Distilled water	-	1000 ml

- **Alkali transfer buffer**

NaOH	-	16 g (0.4 N)
Distilled water	-	1000 ml

- **Prehybridization and Hybridization solutions**

1M Na_2HPO_4 (pH 7.2)	-	10ml (0.5 M)
20% SDS	-	7ml
Distilled water	-	20ml

- **1 M Na_2HPO_4 (pH 7.2)**

  Dissolved 14.1 g Na_2HPO_4 in 100ml distilled water and pH adjusted to 7.2 with Orthophosphoric acid.

- **Post Hybridization washing solution I**

1M Na_2HPO_4 (pH 7.2)	-	2.5 ml
20% SDS	-	12.5 ml
Distilled water		50 ml

- **Post Hybridization washing solution II**

1M Na_2HPO_4 (pH 7.2)	-	2.5 ml
20% SDS	-	2.5 ml
Distilled water	-	50 ml

MIX. For radioactive southern hybridization:

- **20xSSC(for 100ml)**

NaCl	-	17.50 gms.
Sodium citrate	-	8.82 gms.

- **Prehybridisation solution:**

  6x SSC

  0.5%SDS

  5x Denhardt's solution

  100µg denatured salmon sperm

- **Denhardt's Solution:**

Ficoll	-	0.5gms
PVP	-	0.5gms
BSA	-	0.5gms
Water	-	50ml.

This was then kept for 24 hrs in the refrigerator and then filter sterilized.

- **Hybridisation mixture:**

Labelling buffer	-	10 µl
Mixture of unlabelled dNTPs	-	2 µl (20 µM each)
Denatured DNA template	-	500 ng/ml.
Nuclease free BSA	-	400 µg/ml.
αP^{32}dNTP (3000 Ci/mmol)	-	5 µl (333 nM)
DNA Pol I (large klenow fragment)	-	5 units

  Make the volume with nuclease free water up to 50 µl.

- **Promega Packagene @ Lambda DNA Packaging system**

  Components:

6x1 extracts	Packagene system
1x10 µl	Packagene Control DNA
1x0.5 ml	Bacterial strain LE 392.

MX. Methods

MX. 1. Maintenance of *streptomyces* strains

Streptomyces strains used in the study were preserved as mycelial or spore suspensions at -20^{0}C in 20% glycerol. Cultures used frequently were maintained at 4^{0}C upto one month as growth on SMA.

MX. 2. Maintenance of *E. coli* strains

E.coli strains were stored for routine use in refrigerator as cultures on LA plates containing appropriate antibiotics. For long term storage, cultures were preserved in 20% glycerol at -20^{0}C.

MX. 3. Maintenance of bacteriophages

For routine use, lysates of λ clones prepared from confluent plates were stored in refrigerator over a few drops of chloroform.

MX. 4. Growth Condition for *Streptomyces*

Streptomyces were grown at 30° C on orbital shaker at 200 rpm and stored at -20° C.

MX. 5. Growth condition for *E coli*

E coli containing pKC505 plasmid were grown at 30° C and on orbital shakes at 200 rpm and stored at -20°C.

MX. 6. Titration of lambda phage

- Bacterial cultures (host strains) were grown to an OD_{600} of 0.6.
- Phage lysates were diluted serially and appropriate volume of bacterial suspension was mixed with 0.2ml of lysate.
- After incubation for 20 min at 37 °C, 4ml of soft agar was added to each tube and the mixture was overlaid on bottom agar plates.
- Plaque forming units (PFU) were calculated after overnight incubation at 37 °C.

MX. 7. Plasmid isolation (Alkali lysis method)

- Resupend Bacterial pellet, obtained from 10 - 30ml culture, in 1 ml chilled solution I (GTE).
- After 5 min. incubation, add 2 ml of fresh solution II (alkaline SDS).
- Immediately mix the solution very gently and add 1.5 ml of ice-cold solution III (potassium acetate) to it.
- Rotate the contents of the tube about 20 - 25 times and maintain on ice for 10 min.
- Spin the contents at 4000 rpm for 10 min.
- Take the supernatant and treat it first with equal amount of phenol: : $CHCl_3$ – isoamyl mixture and then with $CHCl_3$: iso amyl alcohol. Spin at 5000 rpm for 5 min
- Take supernatant add equal volume of isopropanol. Mix gently and keep it for 15 min. at RT.
- Pellet down at 8000 rpm for 15 min. Wash the pellet twice with 70% ethanol.
- Completely air dry the tube and dissolve the pellet in it. (Molecular cloning a lab manual 3rd addition, volume 1.,Sambrook et al, 2001)

MX. 8. Total DNA isolation of *Streptomyces* (by Hopwood et al 1985)

- Suspend 1 g mycelium in 5 ml TE add lysozyme 10 mg (i.e. to 2mg / ml).
- After one wash with 10.3 % sucrose. Incubate at 30° C.
- Triturate at every 15 min, until a drop of suspension on a microscopic slide is completely cleared by addition of a drop of 10 % SDS.
- Add 1.2 ml 0.5 M EDTA (i.e. to 0.1 M) ; mix gently and incubate at 30° C for 5 min.
- Add 0.7 ml 10 % SDS (to 1 %) tilt immediately , incubate at 37° C for up to 2 hrs.
- Add 6 ml of Tris – saturated phenol, and mixed for 10 min at RT.
- Add 6 ml of $CHCl_3$ and shake for 5 min. at RT.
- The aqueous phase after spin (3000 rpm for 10 min.) was carefully removed with a 1 ml cut tip and re-extracted with phenol: chloroform
- Above followed by two extraction with equal volume of $CHCl_3$.

- DNA was precipitated by adding 1/10 volume of 5 M Na acetate and equal volume of iso-propanol and washed with 70 % ethanol;
- Air dried and resuspended in TE.

MX. 9. Preparation of competent cells

- Competent cells for electroporation were prepared as described by Sambrook et.al, 2001, with minor modifications.
- Fresh overnight culture was grown in LB and then 100µl of this culture was added to 100ml of 2xYT and grown to an OD_{600} of 0.6.
- The cells were chilled on ice and centrifuged at 4000 rpm for 10 min. at 4°C.
- The pellet was resuspended in 100ml of TDW,
- Centrifuged at 4000 rpm for 10 min.,
- Followed by resuspension in 80ml, 40ml TDW, 20ml 10% glycerol,
- Followed by centrifugation each time at 4000 rpm for 10 min.
- After decanting the 10% glycerol, the pellet was dissolved in the residual glycerol
- Aliquots (60µl) were stored in sterile microfuge tubes at -70 °C.

MX. 10. Electroporation *E. coli*

- An aliquot of frozen cells were thawed on ice.
- 100 ng DNA was mixed with the cells and kept on ice for 5 min.
- This suspension was transferred into an electroporation cuvette (0.1cm width; Bio-Rad) and electroporated using the following pulse conditions; voltage, 1.8kV/ 2.2kV; resistance, 200Ω; capacitance, 25 µF, which gives a time constant of 4.5 to 5.5 sec.
- The electroporated cells were diluted with 1 ml LB and kept at 37^0 C for 45 min for expression of antibiotic resistance.
- The culture was then plated on appropriate antibiotic containing LB plates, incubated at 37^0 C for transformats to appear after 12 to 24 hours.

MX. 11. Transformation of *E. coli* (using calcium chloride method) as described by (Sambrook et al, 2001).

- Fresh overnight grown cultures in LB was sub- cultured (1:10) in LB and grown to an $O.D._{600}$ of 0.6.
- Cells were spin down at 5000 rpm for 5 min. at 4°C.
- Supernatant was discarded and the pellet suspended in last drop.
- Add 0.1 M $MgCl_2$ and keep the tube in ice for 10 min., spin for 5 min. at 5000 rpm at 4° C. Discard the Supernanent and resuspended the pellet in 0.1 m calcium chloride (chilled).
- Keep in ice for 10 min. , spin for 5 min. at 5000 rpm 4° C.
- Discard the supernatant , resuspend the pellet in last drop.
- Incubate in ice for 30 min. and add 5-7 µl of DNA mix the content by tapping the tube. Incubate further for 30 min. in ice.
- Give hot shock treatment to cells at 37-42° C for 90 Sec, rapidly transfer to ice bath.
- Add LB and incubate for 45 min. at RT.
- After incubation period the tube was spun down for 5 min at 3000 rpm, supernatant was discarded and the pellet was suspended in last drop.
- The culture was then plated on appropriate antibiotic containing LB plates, incubated at 30° C. The transformants were counted after 12-24 hours.

MX. 12. Transformation *(Streptomyces)*

MX. 12.1. Preparation of protoplasts (Hopwood et. al., 1985)

- Grow the culture in 10 ml medium at 30° C on orbital incubator shaker (170 rpm) for 36 – 40 h.
- Spin and transfer the pallet to 50 ml medium, grow for 28 h.
- Spin the culture and discard the supernatant, give the pellet two washes of 10.3 % sucrose.
- Resuspend mycelium in 4 ml P buffer containing 4 mg lysozymes (at 1 mg/ml).
- Triturate at an interval of every 15 min. After protoplastation is over add 4 ml P buffer,
- Filter the contents through cotton assembly.

- Spin at 3000 rpm for 7 min., give the pellet 2 washes of P buffer and resuspend the pellet in last drop.

MX. 12.2. Preparation of protoplasts (Okanishi et al 1987)

- One ml of a frozen culture of *Streptomyces* was diluted into 9ml of TSB broth and grown for 18hr aerobically at 29°C.
- The culture was homogenized and 5ml was transferred into 45ml of fresh TSB broth supplemented with 0.8% glycine and grown for 16hr at 29°C.
- This culture was homogenized and spun.
- The supernatant was discarded, and the pellet was resuspended in 4ml P-buffer containing 4 mg lysozymes (at 1mg/ml) after two washes of 10.3% sucrose.
- Triturate at an interval of every 15 min.
- After protoplastation is over add 4 ml P buffer, filter the contents through cotton assembly.
- Spin at 3000 rpm for 7 min., give the pellet 2 washes of P buffer and resuspend the pellet in last drop.

MX. 12.3. Transformation of protoplasts

- Dispense 50 µl of protoplasts into as many tubes as there are transformations.
- Add up to 10 µl DNA solution to protoplasts and mix by tapping, immediately add 200 µl of T-buffer (0.25g PEG in 750µl of T-buffer) and mix by pippeting up and down three times.
- Spread the suspension on R_2YE plates.
- Incubate the plates at 30° C. After 14-20 h overlay with soft agar containing antibiotic.
- Score for resistant colonies after 3 days.

MX. 12.4. Spot-transformation-

- Spread 50 µl of protoplasts on predried R2YE plate.
- Spot 5µl DNA sample in TE (20 samples can be spotted on one plate). Add 10 µ lit of PEG in T buffer; dry for 30 min, incubate for 16 hrs and apply antibiotic overlay.
- Transformant colonies may appear after 3-5 days of incubation at 28-30 ^{0} C.

MX. 12.5. Preparation of single stranded DNA for protoplast transformation by denaturation by alkali:

- 2 μ lit of 1 M NaOH added to 9 μ lit of DNA (1-5 μ gms), was mixed by tapping and incubated at 37 ^{0} C for 10 min and rapidly chilled on ice.
- 2 μl of 1 M HCl was added, and the contents were stored on ice till use.

MX. 13. Transduction – λ lysate prepration

MX. 13.1. Broth Method

- Scoop colonies from plate and suspend it in 0.5 ml LB taken in an eppendorf to an $O.D._{600}$ of 0.6. Add $MgCl_2$ 10 mM (2.5 μl).
- Take 0.3 ml of the suspension and add it to an empty sterile flask with cotton plug.
- Add 10 μl of λ lysate in the same flask. Incubate for 20 min. at RT.
- Add 10 ml LB with $MgCl_2$ 10 mM (50 μl), keep the system on shaker at 150 rpm and see intermittently until clear solution is visible.
- First growth will increase and then decrease.
- After lysis is observed spin down the culture, take the supernatant in fresh autoclaved sterile tube.
- Add $CHCl_3$, vortex and spin.
- Take the supernatant in fresh sterile autoclaved tube and repeat the above step of chloroform treatment trice (to kill the cells).
- Store the supernatant at 4° C after adding $CHCl_3$.

MX. 13.2. Agar Method:

- Grow host strain to an $O.D_{600}$ of 0.6.
- Take 1 ml of the culture add $MgCl_2$ 10 mM (5 μl). Use 0.3 ml of this suspension and add 10 μl of λ lysate.
- After incubation for 20 min. at RT, 4 ml soft agar was added and then the mixture was overlaid on bottom agar plate with $MgCl_2$ and antibiotic selection for host strain.
- Plaque forming units (PFU) was calculated after overnight incubation at 30° C.

- Add 3 ml of SM buffer or LB with $CHCl_3$ in the plate, swirl the plate and keep it for 3 hrs. Collect the aqueous phase and spin it down.
- Take supernatant add few drops of $CHCl_3$,
- vortex and spin down.
- Repeat the step of chloroform treatment twice. Finally add chloroform to the aqueous phase and store at 4^0C .

MX. 13.3. Transduction:

- Take one ml of overnight grown culture; add 100 µl of λ lysate and 10Mm $MgCl_2$.
- Process one control tube with no λ lysate added.
- After 20min incubation at RT, spin down the cells and resuspend them in the last drop of LB.
- Wash twice with 5ml LB+5mM sodium pyrophosphate.
- Finally resuspend the pellet in 5ml LB+5mM sodium pyrophosphate.
- Keep the tubes at 30^0C for one hour.
- Pellet down the cells at 5000rpm for 5 min.
- Resuspend the pellet in the last drop of LB and spread on LA plate with 5Mm sodium pyrophosphate and proper antibiotic selection.
- See the transductants grown after 16-20 hour.

MX. 14. Conjugation:

MX. 14.1. Between *E.coli* and *E.coli*

- The donor and recipients cultures are grown in LB to a density of 3×10^8 cells / ml (A600 = 0.7 – 1.0 or 40-60 klett units).
- For the mating process to occur, 4 ml of the donor is transferred to 10 ml of the recipient, mixed properly.
- The conjugation mixture is allowed to stand at $37^0C$for 100 min.
- After the incubation period, 0.1 ml of the mixture is spread on LA plate against proper antibiotic selection.
- The plates are incubated at 30° C for 2 days. Now examine the ex-conjugant.

MX. 14.2. Between *E.coli* and *Streptomyces*

- One ml of a frozen mycelia culture of *Streptomyces* was diluted into 9 ml of TS broth (Baltz, 1978) and incubated for 18hrs aerobically at 29°C.
- The culture was homogenized (Baltz, 1978) and 2 ml was transferred into 18ml of fresh TS broth and grown for 16hrs at 29° C, to obtain a late log phase culture.
- This culture was homogenized and 1 ml was transferred to 9 ml TS broth.
- The culture was incubated aerobically at 37°C for 3 hrs.
- The mycelium was recovered by centrifugation, washed once in TS broth and resuspended in 2ml TS broth (recipient culture).
- The *E. coli* donor, S17 (pKC505 with insert), was grown overnight at 33°C in TY broth plus 50 ug Apramycin/ml, sub cultured 1:100 and grown further for 3 hrs at 33°C.
- The cells were pelleted, washed once in TS broth and resuspended in 2 ml TS broth (donor culture).
- Equal volumes of the donor culture and ten-fold serial dilutions of the recipient culture were mixed, and 100µl was plated onto AS1 (Baltz, 1980), supplemented with 10mM $MgCl_2$.
- Plates were incubated at 30°C for 16 hrs, and then covered with 3-4 ml of sterilized distilled water containing 1-5 mg nalidixic acid and 50 ug/ml of Apramycin.
- Incubation at 30°C was continued for about a week to allow outgrowth of the exconjugants.

MX. 14.3. For control

(a) 100µl of the recipient culture alone selected for Apramycin resistance (negative control)

(b) 50 µl of recipient culture, no Apramycin.

MX. 15. Extraction of antibiotic:

- R_2YE plates were streaked with the culture and incubated at 30° C for approximately one week.

- The agar was finely chopped and immersed in ethyl acetate in flask, shaken on orbital shaker at 170 rpm at RT for two hours; supernatant was collected in a second flask.
- The process repeated twice using fresh ethyl acetate each time.
- Supernatant was evaporated under vacuum to reduce its volume to 0.5 ml in a rotary evaporator to be subsequently used in TLC and bioassay.

MX. 16. Thin layer chromatography:

- A 3 mm thick glass plate was prepared with 1 mm thick silica in distilled water, dried overnight and then baked for 2-3 hours at 60-65° C before use.
- Ready to use silica plate, Silica gel HF_{254} cast on aluminum sheet was also used when fluorescent compound had to be visualized.
- The samples were loaded in the form of a spot, approximately 1.5 cm from edge, and then allowed to dry.
- The plates were then run in appropriate solvent system, air-dried. Separated bands were then visualized with UV – 302 nm wavelength. Bioactivity was checked by development of bioautogram (Usdin et al, 1954) using *Micrococcus luteus* as the test organism.

MX. 17(a). Agarose gel electrophoresis:

- Agarose gel electrophoresis was carried out in a horizontal matrix of agarose with 1X TAE buffer as described by Sam brook et al, (2001).
- Required amount of agarose (depending on the percentage) was taken in conical flask (2 to 4 times the volume of the solution) containing 1x TAE.
- The agarose was melted in a microwave/ boiling waterbath.
- Ethidium bromide solution (0.5 μg/ml) was added for staining the DNA bands.
- The agarose solution was then poured on the gel template sealed with tape and fitted with comb.
- The agarose was allowed to set to gel for 30 min.
- The comb was then removed and the gel was immersed in 1x TAE buffer in horizontal electrophoresis tank.

- The DNA samples were mixed with 1/6 volume of 6x loading buffer and electrophoresed at 5V/cm.
- Lambda DNA digested with *Hind*III/ *Hind*III- *Eco*RI was run in parallel as a size standard.

MX. 17(b). Staining the gel:

- If ethidium bromide was not added in the agarose gel, after electrophoresis, the gel was stained using ethidium bromide solution (0.5 μg/ml) for 30 min.
- The gel was then destained in water.
- The bands were visualized using short wave UV light (302 nm) on a transilluminator.

MX. 17(C). Elution of DNA from agarose gel:

- The digested DNA containing the fragment to be eluted was separated on 0.7 % agarose gel.
- The bands corresponding to desired DNA fragment was excised out and chopped into small pieces, transferred in to 0.5 ml. Eppendorf. With a hole at its bottom blocked by glass wood in TE, and placed into eppendorf of volume 1.5 ml.
- The assembly is kept at -20° C for 5-6 hrs and then spun after thawing at 12,000 rpm for 10 min.
- The DNA was extracted with phenol, precipitated by ethanol, washed with 70 % ethanol, air dried and re suspended in small volume of water or TE.

MX. 18. Bioactivity tests:

MX. 18.1 Bioautograms (Usdin et al, 1954):

After separation of the samples on TLC plate, the separated spots were checked for bioactivity. For this:

- MBA soft agar containing *Micrococcus luteus* was overlaid on the TLC plate and kept overnight for incubation at 30° C.

- Spraying TTC developed the bioautograms. Multiplying cells have dehydrogenase, which reduces TTC into red formazon. Inhibition is observed as clear zone against red background.

MX. 18.2. Agar well assay:

- Melted media was poured in petri plate after solidification soft agar containing test organism was overlaid onto it.
- Well(s) were made with cork borer after which samples were applied and visualized using TTC or either by observing clear areas against the opaque one.

MX. 18.3. Test organism:

Gram positive bacteria	**Media**
Micrococcus luteus	MBA soft
Staphylococcus aureus	MBA soft
Bacillus subtilis	MBA soft

MX. 19. HPLC analysis of mycelia extract:

Analytical HPLC of the mycelia extracts from wild type strain *S. flaviscelroticus* mutant DH. The extracts were loaded on C_{18} column (250mm x 4.6mm) and was developed using gradient run (solvent A – H20 + 0.1% TFA, Solvent B- ACN +0.1% TFA). The run was carried out for 40 min. and the flow rate was 0.5 ml/min. The detection was carried out by UV absorption at 280 nm. The gradient program was as follows:

TableM.3: describing the gradient HPLC run profile:

Program - 1		Program - 2	
Time (In min.)	Concentration of Solvent - B	Time (In min.)	Concentration of Solvent - B
0	0	0	0
5	40	5	40
40	100	10	50
45	0	50	75
		55	100
		60	0

MX. 20. Southern blotting (non radioactive)

After restriction digestion, DNA was electrophoresed on 0.8% agarose gel, the gel was stained with ethidium bromide and photographed. The gel was soaked in 500 ml of depurination buffer for 15 min. The gel was soaked in 500 ml of depurination buffer for 15 min. The gel was rinsed with water and soaked into denaturation solution followed by neutralization solution and was transferred to Hybond membrane using 10X SSC. (Sambrook et.al, 2001).

Prepration of probe, Prehybridization, hybridization, posthybridization wash and signal development on the X- ray was done according to the instruction provided with the Kit.

MX. 21. Southern blotting using radiolabelled probe (same protocol was used for the colony hybridization*):

- After restriction digestion, DNA was electrophoresed on 0.8 % agarose gel.
- The gel was stained with the ethidium bromide and photographed.
- The gel was then washed twice with the 0.25N HCl at room temperature for 15'
- Denature the DNA by soaking the gel in several volumes of 1.5M NaCl and 0.5 M NaOH for 1hour at room temperature with constant stiring and shaking.
- Neutralize the gel by soaking it in several volumes of a solution of 1M Tris-Cl (pH 8.0) and 1.5 NaCl for 1 hour at RT with constant stirring.

- The gel was then kept in 10x SSC
- The gel was kept on the 3MM whatman filter paper in the inverted postion and Hybond nitrocellulose membrane was kept over it with one end cut.
- The DNA transfer was allowed using the 10x SSC by keeping the wick beneath the gel. The assembly was kept for 15- 16 hrs.
- The membrane then taken out and kept between two wahtman paper sheets folded properly in the aluminium foil and then kept at 80^0C for DNA crosslinking.
- Float the membrane containing the target DNA on the surface of the tray of 6x SSC until the membrane becomes thoroughly wetted from beneath. Submerge the membrane for 2 mins.
- The membrane was slipped in a heat sealable bag and for each square centimeters 0.2 ml of prehybridisation solution was added to it. And kept at 65^0C for 1-3 hours
- Now add the denatured radioactive probe to the bag containing the membrane along with the hybridization solution.
- The membrane then washed thrice with 2x SSC and 0.1% of SDS and kept at 65^0C for 20 mins.
- This was then followed by the washing with 1xSSC – 0.1% SDS , followed by 0.5x-0.1%SDS and 0.2xSSC- 0.1% SDS for 20 mins at 65^0C.
- Final washing with the 0.1x SSC- 0.1% SDS was given for 30 mins at 55^0C.
- The membrane was then slipped back in to another plastic bag and was incubated for 1hr - 12 hrs at RT against the X-ray film.
- The x-Ray film was then washed to develop the blot.

* For colony hybridization in stead of gel the agar from the plate on which defferent clones were streaked was used above which the nitrocellulose membrane was kept once the colonies were transferred on the membrane it was dried under vaccum at 80 ^{0}C and then treated with the radiolabelled probe. The subsequent washing procedure carried was the same as it has been described above for the gel extracted DNA hybridization.

MX. 22. Sucrose density gradient of *Sau*3AI partially digested genomic DNA.

- The 1 µg of chromosomal DNA of *S.flaviscleroticus* was partially digested with restriction enzyme *Sau3A* I.
- Four tubes were taken each containing 1 µg of genomic DNA and in each tube different concentration of *Sau3A* I enzyme were used to carry out the digestion.
- These four tubes were incubated at 37°C for 1 hr in order to carry out the partial digestion. The reaction was stopped and twice the DNA was extracted using Phenol and chloroform mix.
- This was then precipitated in 100% ethanol and then dissolved in 200 µl of TE.
- The samples were then size fractionated on continuous sucrose gradient.
- To fractionate these DNA the sucrose gradient of 10% and 40% (w/v) was prepared which was then filter sterilised through 0.2 mm micro filter.
- This was then kept at 4°C for 2-3 hrs at a quite place.
- Then the DNA sample digested with the enzyme and heated at 68 °C for 10 mins and then cooled at 20 °C was then layered gently on the top of the sucrose gradient.
- The tubes were then kept for centrifugation at 25,000 rpm for 22 hrs at 20 °C.
- The tubes were then punctured at the bottom and 300 µl of samples were collected in different tubes.
- Out of each tubes some samples were taken out and was loaded on the gel to see the amount of DNA recovered after the centrifugation.

For the packaging of the ligated cosmid following protocol was used

- The cosmid here was first linearised with HpaI and treated with CIP to avoid the self ligation then was digested with *Bam*HI.
- Genomic DNA fractionated by density gradient was ligated to cosmid vector DNA at stoichiometry of 2:1.
- 1.0µg of the ligated DNA was added to *in vitro* packaging extract (Promega), incubated at 22^{0}C for 2.5 hrs, clarified with chloroform
- Added to 5 ml of host MC1061 cells grown in the presence of 0.2% maltose + 10mM $MgCl_2$ to an OD of 0.5.

- After 30 min at room temperature, 20 ml of Luria broth was added to the infected cells and incubation continued at 30^0C for another 1.5 hrs. 0.1 ml of cells was plated on LA supplemented with 25 μg/ml of apramycin.