Chapter-2

Materials & Methods

MATERIALS

2a.1 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. All other chemicals used in this study were also of analytical grade and obtained from local sources.

2a.2 Microbial Strains: All the microbial strains used in the study were either procured from culture collection or were obtained as gift from various authentic sources.

Table- 2a.1: List of microbial strains used in the study:

Strains	**Genotype / phenotype**	**Source**
	***Streptomyces* strains**	
S.flaviscleroticus	PKS^+ Produces yellow colored diffusible pigment	MTCC
S. lividans TK24	Str-6, $SLP2^-$, $SLP3^-$	MTCC
S. griseus sub griseus	Producer of type II polyketide – Chromomycin A3	Dr. Rosazza Uni. Iowa, USA

***E. coli* Strains**		
DH5α	*deoR, endAl, gyrA96, hsdR17 (rk^- mk^+), recAl, supE44, thi -1,* Δ *(lac ZyA – arg F),* U169, Φ80*dlacZ*ΔM15.	Lab Collection
S17.1	*$recA^-$, mob^+ , thi, pro, $hsdR^-$, $hsdM^+$*/RP4-2-Tc::Mu-Km::Tn*7*	Lab Collection
JP 3301	F^+ *, purE, trp, hTis, argG, ilv, leu, met, thi, ara, xyl, mtl,* Δ*(arg*F*-lac)*U169*, pheR372, recA56[*λ *p(pheA-lac)]*	CCMB, Hyderabad
GJ 1883	*tet^r , rec^- , F^-*	CCMB, Hyderabad
Other strains		
Micrococcus luteus	produces shiny yellow colored colony ATCC4698	IMTECH, Chandigarh
$Chromo^r$	Chromomycin resistant adaptive mutant	This study
Ery^r	Erythromycin resistant adaptive mutant	This study
Tet^r	Tetracycline resistant adaptive mutant	This study
Staphylococcus arueus	isolate	Microbiology Dept.
	BB255	Prof. Brandenburg
	BB270	Prof. Brandenburg
Bacillus subtilis	-	Lab collection
Salmonella typhimarium	Isolate	Microbiology Dept.
Aspergillus flavus	Laboratory isolate	Botany Dept.
Aspergillus niger	Laboratory isolate	Botany Dept.
S. cerevisiae	Isolate	Microbiology Dept.

2a.3 Plasmids and Constructs: Clones were constructed using plasmids available in lab collection or were kindly provided on request, free of cost, from John Innes Centre, UK.

Table- 2a.2: List of plasmids and constructs used in the study

Plasmid	Size (kb)	Features	Source
pBSK	2.94	Ap^r, lacZ , ColE1 ori, T3-T7 promoter, f1	Lab collection
pSET152	5.5	Am^r, *att-int*, oriT, lacZ	Lab collection
p17EBSK	20	17kb EcoR1 insert of *S. flaviscleroticus* PKS cluster cloned in pBSK	Previous work in lab
p11EBSK	14	11kb EcoR1 insert of *S. flaviscleroticus* cloned in pBSK	Previous work in lab
p8EBSK	11	8kb EcoR1 insert of *S. flaviscleroticus* cloned in pBSK	Previous work in lab
p4EBSK	7	4kb EcoR1 insert of *S. flaviscleroticus* cloned in pBSK	Previous work in lab
p5EBSK	8	5kb EcoR1 insert of *S. flaviscleroticus* cloned in pBSK	Previous work in lab
p2.2BBSK	5.2	2.2kb BamH1 fragment containing *actRp* and SflRI cloned in pBSK	Previous work in lab
p11ESET	16.5	11kbEcoR1 fragment of S. flaviscleroticus cloned in pSET152	Previous work in lab
p11EBT	17	p11EBSK fused with pSETΔ*pst* in *pst*I	This study
pIJ8600	8.1	pSET152 derivative with tipAp, $thio^r$	John Innes Centre, UK

pIJ8660	7.7	pSET152 derivative with promoterless EGFP, thio^r	John Innes Centre, UK
pIJ8655	9.5	Am^r	John Innes Centre, UK
pTRACT	9.2	Fusion of HXpBSK with pIJ8600ΔH in EcoR1	This study
HXpBSK	3.7	0.7kb truncated sflRI cloned in HindIII – Xho1 sites of pBSK	This study
pIJ8600ΔH	5.5	att $^-$, int$^-$ derivative of pIJ8600	This study
pOAX	11	p17EBSK digested with Kpn1 and self ligated to reduce insert to 8kb: sfrB$^-$, sfrA$^+$, sfrX$^+$	This study
pBAX	13	p17EBSK digested with Xho1 and self ligated to reduce insert to 10kb: sfrB$^+$, sfrA$^+$, sfrX$^+$	This study
pBAX1	18.5	Fusion of pBAX with pSET152 at EcoR1: sfrB$^+$, sfrA$^+$, sfrX$^+$	This study
pOAX1	16.5	Fusion of pOAX with Pset152 at EcoR1: sfrB$^-$, sfrA$^+$, sfrX$^+$	This study
pBAO1	18.5	Fusion of BglII digested pBAX with pSET152 in BamH1 site: sfrB$^+$, sfrA$^+$, sfrX$^-$	This study
pOAO1	16.5	Fusion of BglII digested pOAX with pSET152 in BamH1 site: sfrB$^-$, sfrA$^+$, sfrX$^-$	This study

2a.4 Enzymes:

Restriction enzymes and T4 DNA Ligase were purchased from Promega, Madison, USA; Roche Molecular Biochemicals, Mannheim, GBR Bangaloregenei, India

Appropriate buffers and BSA provided with restriction enzymes were used.

RNase was purchased from Sigma chemical Co., St. louis, USA.

2a.5 Antibiotics: All antibiotics used were either procured from Sigma Chemical Co. U.S.A. or Hi-media, India and was of analytical grade.

Table- 2.3: Antibiotics used in the study

Antibiotic	Solvent	Concentration (stock)
Ampicillin	Water	100mg/ml
Apramycin	Water	100mg/ml
Cabrenicillin	Water	100mg/ml
Chloramphenicol	Methanol	40mg/ml
Chromomycin	Methanol	100ug/ml
Ciprofloxacin	Water	10mg/ml
Daunomycin	Water	10mg/ml
Erythromycin	70% Ethanol	100mg/ml
Gentamycin	Water	100mg/ml
Hygromycin	Water	50mg/ml
Kanamycin	Water	100mg/ml
Mitomycin	Water	10mg/ml
Nalidixic acid	1N NaOH	30mg/ml
Rifamycin	Methanol	50mg/ml
Spectinomycin	Water	100mg/ml
Streptomycin	Water	100mg/ml
Tetracycline	70% Ethanol	50mg/ml
Thiostreptone	DMF	50mg/ml
Trimethoprim	DMF	60mg/ml

2a.6 Primers: Primers were designed using online free software - PRIMER3 (http://frodo.wi.mit.edu/). Oligonucleodites were procured from MWG-Biotech AG, Germany.

Table- 2.4: List of Primers used in PCR studies

Name	Sequence	Oligo Size	Tm	RE site
Fproact	TAT GGA TCC TTG AGC GGC GGT GGA GA	26mer	68.0°C	*Bam*HI
Rproact	TAT T CT AGA GGG CAG TTC GTG TGT CA	26mer	64.8 °C	*Xba*I
Fact	GCG AAG CTT CAC ACG ATG ACG ATC TCA	27mer	66.5 °C	*Hind*III
Ract	TAT GGT ACC AAC GCA CAC GGA ATC GA	26mer	64.8 °C	*kpn*I
T3	AAT TAA CCC TCA CTA AAG GG	20mer	53.2 °C	-
T7 promoter	TAA TAC GAC TCA CTA TAG GG	20mer	53.2 °C	-
Fwd 16S	AGA GTT TGA TCC TGG CTC AG	20mer	59.4 °C	-
Rev 16S	GCT CGT TGC GGG ACT TAA CC	20mer	59.4 °C	-
Fwd M13	GTA AAA CGA CGG CCA GT	17mer	52.8 °C	-
Rev M13	CAG GAA ACA GCT ATG AC	17mer	50.4 °C	-

Alphabets underlined represents restriction sites

2a.7 Growth media:

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

2a.7.1 Culture Media for *Streptomyces* (Keiser et al, 2000)

Solid media

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

Sucrose	-	10.30 gm
K_2SO_4	-	0.25 gm
Glucose	-	10.00 gm
Difco casamino acids	-	0.10 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

$CuCl_2 . 2H_2O$	-	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

R_2 medium (Hopwood and Wright, 1978)

Same as R_2YE 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	-	8.0 g
Agar	-	0.75 %
pH	-	7.2

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

2a.7.2 Media used for culturing *E.coli*

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.

Luria Broth (LB)

same as LA without agar-agar

Nutrient Broth (NB)

Nutrient broth powder - 8 g / 1000ml distilled water

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	-	12.54g (0.72M)
D/W	-	100ml

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

2a.8 Reagents for plasmid preparation (Sambrook et al, 1989)

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

Note: 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 used cold.

Buffered phenol

Phenol obtained commercially was distilled at 160^0C and stored at 4^0C 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 4^0C.

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.

2a.9 Buffers

P (protoplast) buffer (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 %)	-	1.0 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.

2a.10 Solutions for agarose gel electrophoresis (Sambrook et al, 1989)

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.

2a.11 SDS –PAGE:

30% acrylamide mix

Acrylamide	29% (w/v)
N,N'-methylene-bis –acrylamide	1% (w/v)

10%SDS

10% ammonium persulphate(APS)

TEMED

Water saturated n-butanol

Buffers

Lysis buffer

Tris	20mM
Triton-X 100	0.1%
Lysozyme	0.2%

Stacking gel buffer	0.5M Tris(pH 6.8)
Separating gel buffer	1.5M Tris (pH8.8)

Tank buffer

Tris	25mM
Glycine	250mM
SDS	0.1%
pH	8.3

5x SDS gel loading buffer

Tris-Cl (pH 6.8)	100mM
SDS	4%(w/v)
Bromophenol blue	0.2%(w/v)
Glycerol	50%(v/v)
β-mercaptoethanol	500mM

Staining solution

Coomassie blue	2.5%
Methanol	40%
Glacial Acetic acid	7%

Destaining solution

Methanol	50%
Glacial acetic acid	10%

2a.12 Miscellaneous:

Solvent system for TLC

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

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

DNA loading Dye (6X)

0.25% (w/v) Bromo phenol blue
60% (w/v) sucrose
100mM EDTA pH 8.

025% (w/v) Bromo phenol blue
30% Glycerol

Staining: During electrophoresis - add 0.5-1µg/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 Na-acetate (pH 5.2). Heat at 100^{o}C for 15 minutes to remove DNAse contamination, if any. 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^{o} C.

Table- : List of miscellaneous reagents

Solute	Solvent	Stock
Ethidium Bromide	Water	10mg/ml
Reserpine	DMSO	10mg/ml
Diphenyl amine	DMFO	10mg/ml
1,6-diphenyl-1,3,5-hexatriene	Tetrahydrofuran	12mM
IPTG	Water	100mM
X-gal	DMSO	2%

METHODS

2b.1 Culture Maintenance and Growth conditions:

Growth and Storage of *Streptomyces* strains

Streptomyces strains used in the study were grown at 30^{o} C on orbital shaker at 200 rpm and 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 mont as growth on SMA.

Maintenance of *E. coli* strains

E.coli stra were grown at 37^{o}C on obribtal shaker at 200 rpm (unless specified), 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.

2b.2 DNA isolations:

2b.2.1 Plasmid isolation from *E. coli* by Alkali lysis method (Sambrook et al, 1989)

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.

2b.2.2 Genomic DNA isolation of *Streptomyces* (Hopwood et al 1985)

Suspend 1 g mycelium in 5 ml TE add lysozyme 2mg / ml after one wash with 10.3 % sucrose. Incubate at 30^{o} 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^{o} C for 5 min. Add 1 % SDS (final concentration) and tilt immediately, incubate at 37^{o} C for up to 2 hrs. To this mixture, add 6 ml of Tris – saturated phenol and mixed thoroughly at RT. Add 6 ml of $CHCl_3$ and shake for 5 min. at RT. Remove the aqueous phase carefully, after spin (3000 rpm for 10 min.), using a 1 ml cut tip and re-extracted with phenol: chloroform as above followed by two extraction with equal volume of $CHCl_3$. Precipitate DNA 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.

2b.2.3 Total DNA isolation of *M. luteus*

The protocol for DNA isolation of M. luteus was similar to that of *Streptomyces*, with minor changes. Cells were given washes of phosphate buffer instead of 10.3% sucrose and the lysozyme treatment was extended to 1 hr at 65^{o}C. Step involving extraction from Tris-saturated Phenol was repeated twice to eliminate heavy protein content of lysate. Remaining procedure was unaltered.

2b.3 Introduction of DNA into cells:

2b.3.1 Electroporation in *E. coli*

Preparation of competent cells:

Competent cells for electroporation were prepared as described by Sambrook et.al, 1989, with minor modifications. Fresh overnight culture of *E. coli* 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 and aliquots (60µl) were stored in sterile microfuge tubes at -70 °C.

Electroporation

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 suspention 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.

2b.3.2 Transformation of *E. coli* using $CaCl_2$ (Sambrook et al, 1989).

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 spin 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.

2b.3.3 Conjugation Between *E.coli* and *E.coli*

The donor and recipients cultures are grown in LB to a density of 3 X 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°C 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.

2b.3.4 Transformation in *Streptomyces*

Preparation of protoplasts

Method- 1 (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.

Method- 2 (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.

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^o C. After 14-20 h overlay with soft agar containing antibiotic. score for resistant colonies after 3 days.

2b.3.5 Conjugation 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^{o}C. The culture was homogenized (Baltz, 1978) and 2 ml was transferred into 18ml of fresh TS broth and grown for 16hrs at 29^o 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^{o}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 strain - S17.1, was grown overnight at 33^{o}C in TY broth plus 50 ug Apramycin/ml, sub cultured 1:100 and grown further for 3 hrs at 33^{o}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^{o}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^{o}C was continued for about a week to allow outgrowth of the exconjugants.

2b.4 Electrophoresis

2b.4.1 SDS-Polyacrylamide Gel Electrophoresis

2b.4.1.1 Sample preparation

Method 1: Cultures were grown in 10ml LB (150rmp, 37^{o}C) without or with appropriate selection pressure. Since there was difference in growth rates of cultures under different conditions, the cultures were collected at different time points keeping the 0.6OD parameter constant. Cells from each culture set were harvested and were resuspended in 500ul lysis buffer after two washes of phosphate buffer.

Method 2: A 50ul stationary phase culture in broth was used as inoculum to spread on LA plates supplemented with appropriate concentration of selection pressure. Cells were incubated overnight at 37^{o}C. Colonies from plates were harvested and resuspended in 5ml phosphate buffer. OD_{600} of different cultures were measured and the cell concentration was adjusted by phosphate buffer. Equal volume of cell suspension was pelleted and resuspended in 500ul of lysis buffer.
Cells had to be maintained at 37^{o}C for 2 hrs under shaking condition to ensure total lysis. The lysate was sonicated to obtain a clear protein solution.

2b.4.1.2 Gel preparation

Set up gel plates as described in manufacturer's guidelines. Use appropriate spacers and choose a relevant comb with required well size/number. Mix all the reagents separately for appropriate concentration of resolving gel and stacking gel, except APS and TEMED. Then add APS and TEMED to resolving gel, mix and pour immediately about 3-3.5ml per gel. Quickly cover the surface with water saturated Butanol to generate even surface. Once polymerized, remove butanol and rinse it thoroughly with autoclaved distilled water. Now add APS and TEMED to stacking mix and pour it gently on top of the resolving gel through one side of the comb. Stacking gel takes around 15-20 min to solidify. Before removing the comb, mark the wells with a marker. Before loading gels,

wash out wells with distilled water to remove unpolymerised acrylamide. Place the set up in the chamber with running buffer and load samples in the wells using micropipette.

Separating gel preparation (for 10ml)

2.6

Components	Volume (ml)				
	6%	8%	10%	12%	15%
H_2O	3.3	4.6	4.0	3.3	2.3
30% acrylamide mix	2.0	2.7	3.3	4.0	5.0
1.5M Tris (pH8.8)	2.5	2.5	2.5	2.5	2.5
10%SDS	0.1	0.1	0.1	0.1	0.1
10% APS	0.1	0.1	0.1	0.1	0.1
TEMED	0.004	0.004	0.004	0.004	0.004

5% Stacking gel preparation (for 4ml)

Components	Volume (ml)
H_2O	2.44
30% acrylamide mix	0.532
0.5M Tris (pH6.8)	1.0
10%SDS	0.04
10% APS	0.04
TEMED	0.002

2b.4.2 Agarose gel electrophoresis:

2b.4.2.1 Gel preparation

Agarose gel electrophoresis was carried out in a horizontal matrix of agarose with 1X TAE buffer as described by Sam brook et al, (1989). 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.

2b.4.2.2 Staining the gel:

Whenever 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.

2b.4.2.3 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^{o} 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.

2b.5 Antibiotic Extraction and Purification

2b.5.1 Extraction of crude extract:

R_2YE plates were streaked with the culture and incubated at 30^o 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.

2b.5.2 Purification by 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^o 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.

2b.6 Bioactivity tests:

2b.6.1 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^o 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.

2b.6.2 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.

2b.6.3 Growth curve

A 50ml culture of *Micrococcus luteus* was grown on a rotary shaker at 37^{o} C till it reached 0.4 O.D. The culture was equally divided into 4 empty sterilized flasks. Two cultures were grown in presence of polyketide and one in presence of ampicillin- a known lytic antibiotic, maintaining one culture as a control. Following addition of antibiotics, $O.D._{.600}$ was measured on specrophotometer (Helios), at a regular interval of one hour. After one hour of antibiotic exposure, one of the cultures treated with polyketide was transferred to a fresh antibiotic free media, following two phosphate buffer washes to eliminate residual chromomycin. Average data from readings of three independent experiments was obtained and plotted on time vs. OD graph.

2b.6.4 MIC for induced stationary phase cultures (NCCLS guidelines)

For induction of stationary phase, actively growing cells in log phase were harvested and were transferred to phosphate buffer. Following two washes, cells were resuspended in fresh phosphate buffer and maintained for 2hrs at 37^{o}C and 150 rpm. The time was standardized to ensure that the cells had completely consumed all the sugars. Rest of the procedure was same as for MIC determination with appropriate controls.

2b.6.5 Post antibiotic effect (Craig and Gundsmon, 1999)

The log-phase bacteria were collected and adjusted to a concentration of 4 X 10^{8}CFU/ml. After the treatment of the bacterial cultures in the presence of the polyketide

at MIC concentrations for 1 h, the cells were diluted 1:200 in fresh medium and readings were noted spectrophotometrically at regular intervals of 30min. The postantibiotic effect (PAE) was calculated by the standard equation, *T* - *C*, where *T* is the time required for the CFU count in the test culture to increase 10-fold above the count observed immediately after drug removal, and *C* is the time required for the count for the untreated control to increase 10-fold under the same conditions.

2b.7 Generation of adaptive mutants

The minimal inhibitory concentration of *M. luteus* being 120 ng/ml, as obtained in the results of broth assays, the starter culture was setup with an assured sub-lethal dose of 5ng/ml chromomycin in LB medium and incubated at 37^{o}C at 150rpm till culture reached stationary phase. Each time the culture reached stationary phase, an aliquote (1%) was subcultured to medium supplemented with doubled antibiotic concentration and maintained under same conditions. Sixth and subsequent subculturing steps involved an extra step, wherein the cultures were transferred to fresh media with same antibiotic concentration and allowed to grow, before exposing it to higher antibiotic concentration. At every subculture / transfer step, an aliquot was spread on LA agar plates, after appropriate serial dilutions, to ensure purity of cultures.

2b.8 Reserpine assisted inhibition of drug extrusion study (Brenwald et al, 1997)

First the toxicity of reserpine to wildtype and adapted cultures was checked by growing them in increasing concentration ranging from 0.2, 0.5, 2 10, 20 ug/ml. Without death or growth retardation in growth, growth inhibition studies were conducted by a standard broth dilution method. The MICs of pre- and post-adapted *M. luteus* cultures were obtained in the presence and absence of reserpine (2ug/ml). Following the incubation period, bacterial growth was assessed by observing the turbidity of the medium. Experiments were repeated on four separate occasions

2b.9 Membrane fluidity assay (Tourdot-Marechal et al, 2000)

Bacterial cells from a 24 h culture were aseptically harvested by centrifugation at 5000 rpm for 5 min at room temperature and washed twice in sterile phosphate buffer and resuspended in the same buffer to an OD 600 nm of 0.4. One microlitre of the fluorescent membrane probe, 1,6-diphenyl-1,3,5-hexatriene, was added to 3 ml of each resuspended culture in a quartz cuvette and incubated for 10 min in the dark at room temperature to allow probe incorporation into the cytoplasmic membrane. Fluorescence polarization was measured using a spectrofluorometer (Shimadzu RTF750) Excitation and emission wavelengths were set at 360 and 430 nm, respectively. Polarizers were set in either a vertical or a horizontal position. The slit widths for the excitation and emission beams were 5 and 10 nm, respectively.

The degree of polarization (P, unitless) was calculated from the emission fluorescence intensities measured parallel (F1) and perpendicular (F2) to the plane of excitation light according to the following equation:

$$P = \frac{F1-F2}{F1+F2.}$$

2b.10 Nucleic acid binding assay

Based on previous data from our lab (Namita, 2007) the exitation wavelength (470nm) and emission spectra (500-650nm) for the polyketide were fixed with bandpasses of 5nm and 10nm each, to record the data on spectrofluorimeter (Shimadzu RTZ, 750). Chromomycin A3, for which the method was developed earlier (Chakraborty and Dasgupta, 2001) was used as standard. Fluorescence emission spectra were recorded for the polyketide, followed by addition of 10mM Mg^{+2} and finally in presence of 10ug/ml nucleic acid (DNA/RNA) in 20 m*M* Tris-HCl buffer (pH 8.0). Whole process was carried out using standard as well. Fluorscence of each component of mixture was also measured in isolation.

2b.11 Genomic DNA library preparation

Partial digestions of genomic DNA were carried out for 2.5, 5, 7.5 and 10 min. time intervals at 37^{o}C in thermocycler, using frequent cutter restriction enzyme Sau3A. Reaction was stopped by immediately raising the temperature to 94 ^{o}C. Electrophoresis of reaction product with Lambda –Hind marker was carried out in 0.8% agarose gel. Gel corresponding to 5 – 9kb sized fragments was eluted from the gel and was subjected to over night cracking in conventional glass wool assembly at -20 ^{o}C. DNA was retrieved from gel by centrifugation (5000rpm, 5 min, RT). The collected DNA sample was purified and used for ligation with pBSK digested with BamH1. Ligation mixture was introduced in DH5α by electroporation and ligation frequency was determined by blue-white selection. Subsequently the transformants were directly screened on erythromycin to obtain a positive clone.

2b.12 PCR protocols

Step	Time				
	16S	Act	M13	T3/T7	Trunact
Initial denaturation 94 ^{o}C	8:00	2:00	3:00	3:00	4:00
Round 1 (5 cycles)					
Denaturation - 94 ^{o}C	1:30	1:00	1:00	1:00	1:30
Annealing	1:00 (56 ^{o}C)	0:45 (62 - 0.5)	0:45 (56.0)	0:50 (48.0)	0:50 (50- 0.5)
Amplification – 72 ^{o}C	1:30	1:00	1:30	1:30	1:25
Round 2 (25 cycles)					
Denaturation - 94 ^{o}C	-	0:45	-	-	0:50
Annealing	-	0:45 (59.0)	-	-	0:50 (47.5)
Amplification – 72 ^{o}C	-	0:45	-	-	1:25

Final Extention - 72 °C	7:00	7:00	7:00	7:00	7:00
Hold - 20 °C	end	end	end	end	end

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