Chapter 2

Materials and Methods

2.1 Bacterial strains and plasmids used are as listed in Table 2.1

Table 2.1 List of strains and plasmids used

Bacterial Strains		
S. flavisclerotícus	PKS^+ Produces yellow colored antibiotic chromomycin	MTCC, IMTECH, Chandigarh, India
JP1	ΔPKS	This work
INT	PKS^+, #2.19 cosmid integrant of JP1 (Tsr^r and Ap^r)	This work
E. coli (DH5α)	F^- endA1 glnV44 thi 1 recA1 relA1 gyrA96 deoR nupG purB20 φ80dlacZΔM15 Δ(lacZYA-argF)U169, hsdR17($r_K^- m_K^+$), λ^-	Lab Collection
Plasmids		
pKC505	Am^r Streptomyces - E.coli shuttle cosmid	IMTECH, Chandigarh, India
pGM160	7.7kb, Gm^r,Tsr^r, Amp^r	John Innes Center (JIC) , U.K.
pGMΔpst	6kb, Gm^r,Tsr^r,Amp^r 1.7kb DNA between two *Pst*I sites is removed by intramolecular ligation following digestion by *Pst*I	This work
#2.19	Partial chromomycin biosynthesis cluster of PKS genes cloned in pKC505 cosmid selected from genomic library	This work
#2.19ΔBgl	Insert DNA between *Bgl*II restriction enzymes is removed from #2.19 by enzyme digestion followed by intramolecular ligation	This work
#2.19ΔBglIIpGMΔPst	Cloning of pGMΔ*pst* into #2.19ΔBgl at unique *Bgl*II restriction site	This work
pIJ2345	pBR329 with a 2.2kb *actI* insert	John Innes Center, U.K.
pBluescript KS (+)	3kb, *colE1* (derivative) and $F1^{**}$(phage derived) origin of replication, high copy cloning vector, multiple cloning site flanked by T3 and T7 promoters	Stratagene
ET12567	F–dam-13::Tn9 dcm-6 hsdM hsdR zjj-202::Tn10 recF143 galK2 galT22 ara-14 lacY1 xyl-5 leuB6 thi-1 tonA31 rpsL136 hisG4 tsx78 mtl-1 glnV44	JIC, U.K.
S17.1	recA pro hsdR RP42Tc::Mu Km::Tn7 integrated into the chromosome	JIC, U.K.
S. lividans	Used as heterologous host and producer of antibiotics – calcium dependent antibiotic (CDA), actinorhodin (ACT) and undecylprodigeosin (RED)	JIC, U.K.
PKS - Polyketide Synthase; Antibiotic markers: Amp^r – Ampicillin resistance, Tsr^r- Thiostreptone resistance, Ap^r- Apramycin resistance, Gm^r- Gentamycin resistance		

2.2 Growth media and reagent preparation

Media, solutions for media, buffers, and solutions for plasmid preparation, glasswares and plastic wares were sterilized by autoclaving at 15 lbs at 121°C for 20 min., unless otherwise specified.

2.2.1 Culture Media for *Streptomyces*

S. flavisclerotícus and its derivatives were routinely grown on R2YE and TSB medium (HiMedia)(1).

2.2.2 R2YE medium

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 (IO N)	0.03 ml (sterilization not need)

Composition of Trace element solution (for 1000ml)

$ZnCl_2$	40 mg
$FeCl_3.6H_2O$	200 mg
$CuCl_2.2H_20$	10 mg
$MnCl_2.4H_20$	10 mg
$Na_2B_407.10H_2O$	10 mg
$(NH_4)_6\ MO_7O_{24}.4H_20$	10 mg

2.2.3 Soyabeam Mannitol Medium (SM)

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

2.2.4 Tryptone Soya Broth (TSB)

For 1000ml:

Tryptone Soya broth powder - 30 g

Distill water - 1000 ml

2.2.5 Buffered R4 medium (Minimal media)

Composition same as R2YE medium but YE not added. Glucose or casamino acids (0.5%) added as carbon source (1).

2.2.6 Carbon/nitrogen ratio of minimal media:

C/N ratio of R4 media is 9.4, R4 plus CAA is 20.9, R4 plus 5 amino acids (arginine, glutamine, glutamic acid, histidine and proline) is 11.5, R4 plus arginine is 10.68 and R4 plus 15 amino acids (alanine. asparagine, aspartic acid, cysteine, glycine, isoleucine, leucine, lysine, methionine, phenylalanine, serine, threonine, tyrosine, tryptophan and valine) is 13.2.

2.3 Reagents for plasmid preparation

2.3.1 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.

2.3.2 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.

2.3.3 Buffered phenol

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

2.3.4 10 X Tris EDTA (TE), pH 8.0

100 mM Tris –Cl (pH 8.0)
10 mM EDTA (pH 8.0)

2.3.5 Sodium Acetate (3 M, pH 5.2)

Dissolve 408.3 g of $CH_3COONa.3H2O$ in 800 ml of H_2O. Adjust the pH to 5.2 with glacial acetic acid. Adjust the volume to 1 liter with water.

2.4 Buffers

2.4.1 P (protoplast) buffer

Make up the following basal solution:
Sucrose - 103 g
K_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

2.4.2 T (Transformation) buffer

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)

2.4.3 Tris – Maleic acid buffer

Make up a 1M solution of Tris and adjust to pH 8.0 by adding Maleic acid.
Solutions for agarose gel electrophoresis (Sambrook *et al.*, 1989)

2.4.4 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.

2.5 List of antibiotics used are listed in Table 2.2

Table 2.2 List of antibiotics used

Antibiotic	Stock conc.	Working conc.
Ampicillin (Sigma)	100 mg / ml	50-100 μg / ml
Apramycin (Sigma)	100 mg / ml	50 μg / ml
Thiostreptone (Sigma)	50 mg / ml	50 μg / ml
Nalidixic acid (HiMedia Lab)	30 mg / ml	30-40 μg / ml

2.6 Experimental Procedures

2.6.1 Extraction of antibiotic chromomycin

Cultures of wild type, INT and JP1 mutant were plated on equal number of R2YE plates 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; and supernatant was collected in a second flask. The process was repeated thrice 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 HPLC and bioassay.

2.6.2 Testing purity and concentration of chromomycin by HPLC

Analytical HPLC of the mycelial extracts from wild type and JP1 mutant and that of integrant containing #2.19 was carried out on C_{18} column (250 mm x 4.6 mm) and was developed using gradient run (solvent A - H_20 + 0.1% TFA, Solvent B - ACN + 0.1% TFA). The separation was carried out for 40 minutes and the flow rate was 0.5 ml/min. The detection was carried out by UV absorption at 280 nm. Pure chromomycin A3 from Sigma was used as standard during HPLC separation of components of the extracts of different cultures.

The gradient program was as follows:

Time (min.)	Concentration of solvent – B
0	0
5	40
10	50
50	75
55	100
60	0

2.6.3 Southern hybridization and blotting

After restriction digestion, DNA was electrophoresed on 0.8% agarose gel, stained with ethidium bromide and photographed. The gel was soaked in 500 ml of depurination buffer for 15 min., treated with 500 ml of depurination buffer for 15 minutes, rinsed twice with water, soaked in denaturation solution followed by neutralization solution and was transferred to Hybond membrane using 10X SSC (2). Preparation of probe, Prehybridization, hybridization, post hybridization wash and signal development on the X- ray was done according to the instruction provided with the kit (DIG Hi Prime DNA Labeling and detection starter Kit, Roche Diagnostics Ltd.).

2.6.4 Quantitation of growth

Inability of the JP1 mutant to form spores was the reason the mycelial inoculum was used to initiate growth for all the strains. Growth was initiated with 1% viable inoculum of each of the strain and the increase in cell mass was monitored by DNA estimation by diphenylamine method as described by (3), for cells harvested at every 4 hours of interval. Diphenylamine reacts with deoxyribose sugar under acidic condition producing blue-coloured complex measured at 595 nm.

2.6.5 Protein estimation

Protein content was determined using Lowry's method using bovine serum albumin as standard (4).

2.6.6 Viability measurements:

Cells were washed with 0.9% (w/v) NaCl and 20 µl of suspension was used for staining with "LIVE/DEAD Bac-Light Bacterial Viability Kit (Molecular Probes) according to manufacturer instructions, where equal amounts of both culture and dye (mixture of Syto9 and propidium iodide which differentially stain live cells (green) and dead cells (red)) were mixed and kept for 10 minutes for staining and after that a loopfull of cells were taken on slide to observe under Leica TCSSP2-AOBS confocal laser-scanning microscope and LSM – 710 confocal microscope at wavelengths of 488 nm and 568 nm excitation and 530 nm (green) or 630 nm (red) emission (5). Simultaneously, 0.1 ml of cultures was also plated on R2YE agar.

2.6.7 Measurement of minimum-inhibitory concentration (MIC) of chromomycin for JP1

Chromomycin was serially diluted two fold and added to the TSB broth inoculated with 1% volume of freshly grown JP1 mutant. At ~500 $ng.ml^{-1}$ concentration, there was no growth after 48 h of incubation at 30°C and was considered as MIC. A concentration less than the MIC (sub-MIC, 315 $ng.ml^{-1}$) permitted uninhibited growth of the cells and were used for *in-vivo* studies.

2.6.8 Enzyme assay methods

Enzyme assays were carried out from culture grown in TSB (tryptone soy broth) medium. Normalization of the cultures used for all enzyme assays was carried out considering viability and protein estimation. For measurement of enzyme activities of antioxidant enzymes (catalase, alkylhydroperoxidase reductase and SOD), glycolytic and TCA enzymes (PFK, aconitase, G6PDH, 6-PGDH & IDH) and quantitation of metabolites (Acetyl-CoA, α-KG & NADPH) wild type and integrant cells grown for 24-32 h and JP1 cells grown for 40-46 h were selected to represent mid-log phase of growth. For estimation of antioxidant enzyme activities (catalase, SOD and AHP) in stationary phase cultures, WT and INT were grown for 50-60 h and JP1 mutant was grown for 70-80 h. The viability of JP1 mutant at time point i.e. 70-80 h is partial and represents stationary phase of JP1.

Cell pellets were washed with respective assay buffer unless indicated and resuspended in same buffer; cells were disrupted by sonication in ice bath using Branson Sonifier, Model no. 15 for 1 minute at 20% amplitude with 15 seconds on/off

cycle. Cell debris was pelleted down and the supernatant was used for enzymatic assays.

2.6.9 Catalase enzyme activity

Catalase activity was quantified spectrophotometrically by following the rate of decrease in absorbance at 240 nm caused by the disappearance of H_2O_2. The assay mix contained 25-50 µl of protein extract and 10 mM of H_2O_2 in 50 mM phosphate buffer pH 7.4 to final volume of 1 ml. Assay carried out at 25° C. One unit of enzyme activity is defined as the amount required for the conversion of 1µmol substrate into product per minute (6).

2.6.10 Alkyl hydroperoxidase reductase activity

Alkyl hydroperoxidase activity was monitored in a reaction mixture containing 50 mM sodium acetate buffer (pH 5.5), 2 mM H_2O_2 and 1 mM o-dianisidine and by following the rate of oxidation at 460 nm (ε_{460}-11.3 X 10^3 M^{-1}.cm^{-1}). One unit of peroxidase activity is defined as amount of enzyme causing disappearance of 1 pmol substrate min^{-1} (7).

2.6.11 Superoxide dismutase enzyme activity

Superoxide dismutase activity was measured by pyrogallol method. Different volumes of cell supernatant were added to 0.1 mM TrisCl buffer of pH 8.0 followed by 100 µl of 4 mM of pyrogallol. Autooxidation of pyrogallol was monitored by recording the absorbance at 420 nm every 10 sec for 3 minutes. Specific activity of SOD was estimated by units of enzyme required for 50% inhibition of auto-oxidation of pyrogallol per mg protein (8)

2.6.12 Phosphofructo kinase enzyme activity

Cells were harvested and suspended in buffer containing 50 mM TES (N-[tris(hydroxymethyl)methyl]- 2-aminoethanesulfonic acid, pH 7.2, 5 mM $MgCl_2$, 5 mM 2-mercaptoethanol, 50 mM $(NH_4)_2SO_4$ and 0.1 mM phenyl methyl sulphonyl fluoride (buffer A) and disrupted by sonication as detailed above. Different volumes of a assay mixture was added to 50 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 1 mM KCl, 3 mM NH_4Cl, 5 mM dithiothreitol, 0.15 mM NADH, 10 mM fructose-6-phosphate (F-6-P), 0.9 U of fructose biphosphate aldolase, 5 U of triose phosphate isomerase, 0.85 U of α-glycerol-3-phosphate dehydrogenase, and the reaction was started by addition of 2.5 mM ATP for ATP-PFK and 2.5 mM PP_i for PP_i-PFK. Consumption of

NADH is measured at 340 nm. 1 U of enzyme activity is equivalent to 1 µmol of NADH consumed per minute per mg of protein (9).

2.6.13 Aconitase enzyme activity

Aconitase activity was assayed in the extract at 240 nm as the conversion of cis–aconitate to isocitrate, and the specific activity was calculated using an absorption coefficient of 3.6 mM. One unit (U) of enzyme activity converted 1 nmol of substrate per minute; cellular activities are expressed as $U \cdot mg^{-1}$ (10).

2.6.14 Glucose 6-Phosphate dehydrogense activity and 6-Phosphogluconate dehydrogenase activity

Both the enzymes were assayed in a reaction containing 50 µmol of $MgCl_2$, 0.5 µmol of NADP, and 1 µmol of glucose 6-phosphate or 6-phosphogluconate, as described by Ramanov *et. al.,* except that Tris-HCl buffer (50 mM, pH 7.5) was used in the former assay and potassium phosphate buffer (50 mM, pH 7.6) for the latter. Increase in A_{340} /min is monitored for 4-5 minutes. 1 Unit reduces one micromole of pyridine nucleotide per minute at 30°C under the specified conditions (11).

2.6.15 NAD/NADP isocitrate dehydrogenase activity assay

The reaction mixture contained 0.1 M Tris-HCl (pH 7.4), 4 mM $MnCl_2$, 2 mM ADP, 2 mM NADP / NAD, 4 mM isocitrate. One unit (U) of specific enzyme activity was expressed as the amount of enzyme required to convert 1 µmol substrate into specific product per minute per milligram of protein (12).

2.6.16 Estimation of α-ketoglutarate

Cell extract for α-KG (alpha ketoglutarate) estimation was prepared in phosphate buffer saline and sonicated following centrifugation at 15000 x g at 4°C and was used immediately for α-KG estimation. The enzyme assay solution consisted of 100 mM KH_2PO_4 (pH 7.2), 10 mM NH_4Cl, 5 mM $MgCl_2$, and 0.15 mM NADH, the reaction was started by the addition of 5 units of glutamate dehydrogenase. The absorbance decrease was monitored at 340 nm. Enzyme activity was calculated using 6.22 $mM^{-1}. cm^{-1}$ as molar extinction coefficient of NADH (13).

2.6.17 Quantification of intracellular Reactive Oxygen Species

Levels of intracellular ROS were detected by DCFDA (Sigma). Aliquots from the culture broth were taken at selected time points, cell pellets were suspended in 50

mM potassium-phosphate buffer pH 7.4 and DCFDA was added to the final concentration of 1 µg ml^{-1}. Cells were incubated for 30 minutes at 30°C in the dark. Mycelia were washed twice in 50 mM potassium phosphate buffer pH 7.4 and then cells were broken by sonication. The amount of ROS was quantified with a spectrofluorometer (Hitachi Fluorescence spectrophotometer F-7000, excitation at 460 nm and emission at 530 nm). Protein content of crude extracts was used as normalization factor.

2.6.18 Extracellular H_2O_2 estimation

H_2O_2 quantitation was carried out as described by (14) with following modifications. In the assay, horseradish peroxidase (HRP) oxidizes amplex red (AR) dye to the fluorescent product resorufin in the presence of H_2O_2 which is measured at 570 nm. 1 mg of amplex red (light sensitive) was dissolved in 0.78 ml of DMSO, and 0.75 ml of this solution was then diluted into 18 ml of 50 mM potassium phosphate buffer (pH 7.8) to generate a 200 µM stock solution. HRP was dissolved in 50 mM potassium phosphate buffer (pH 7.8) to 0.02 mg/ml. To measure extracellular H_2O_2, 0.5 ml of sample was withdrawn at different phases of growth, washed twice with potassium phosphate buffer (50 mM, pH 7.8), resuspended in 0.5 ml of the same buffer and mixed with 0.25 ml of AR and 0.25 ml of HRP to make final volume 1ml. The mixture was incubated in dark for half an hour. After incubation cells were pelleted down and absorbance of the supernatant was recorded at 570 nm. H_2O_2 concentration was calculated using a curve obtained from standard samples. A small amount of H_2O_2 is generated by the dye/HRP detection system itself; this amount was accounted for by the standard curve. Normalization was done by protein estimation.

2.6.19 Extraction of intracellular NADPH for HPLC

Bacterial cells grown to mid-log phase were suspended in a cell storage buffer (CSB; 50 mM Tris-HCl, 5 mM $MgCl_2$, and 1 mM phenylmethylsulfonylfluoride, pH 7.3), disrupted by sonication and subjected to centrifugation at 3000 x g to remove any intact cells. The cell-free extract (CFE) was then centrifuged for 3 h at 1,80,000 x g to obtain a soluble CFE fraction. Purity of the fraction was verified by monitoring glucose 6–phosphate dehydrogenase activity. For NADPH estimation, soluble CFE was diluted to 2 mg protein equivalent/ml in ddH_2O and then boiled for two minutes. Following removal of precipitate, the supernatant was injected in Agilent 1260

Infinity HPLC equipped with C_{18} reverse phase column (Agilent 5 HC-C18 (2) 250 X 4.6 mm) (15). HPLC protocol for separation and quantitation is described in.

2.6.20 HPLC conditions for NADPH

The mobile phase consisted 0.2 M ammonium acetate and HPLC grade MeOH. The HPLC pumps were initially set at 96% ammonium acetate and 4% MeOH. At 1 min, the MeOH was set to increase by 0.2% per minute and allowed to increase for 25 min. The flow rate was 1 ml/min. The pH was 5.9. Standard NADPH was procured from Sigma, dissolved freshly in triple distilled water at concentration of 1 mg/ml. Various concentrations of standards were injected to get a calibration curve. Samples of CFE of wild type and mutant were injected (16).

2.6.21 NADPH estimation by enzymatic cycling method

NADPH amounts were quantitated in samples by enzyme cycling assay as described (17). A master reagent mix was prepared with 1X Bicine buffer (2.0 M, pH 8.0), 1X 40 mM EDTA, 1X 50 mM glucose 6-phosphate disodium salt, 1X 4.2 mM thiazolyl blue, and 2X 16.6 mM phenazine ethosulfate. The reagent mix was warmed to 30°C. The cycling reaction was carried out by adding 180 µl. of reaction mixture with 30 µl. of extract and 0.6 units of glucose 6-phosphate dehydrogenase from baker's yeast (*S. cerevisiae*) (Sigma no. G7877) by diluting in 0.1 M Bicine buffer (pH 8.0). Water was added to make final volume 300 µl. The change in absorbance after every 30 seconds for 3-5 minutes was read at 570 nm which is the spectral peak of thiazolyl blue that increases upon reduction. Standard curves using NADPH were used to calculate the absolute concentrations in mM and values were normalized by protein estimation.

2.6.22 Determination of intracellular concentration of acetyl-CoA

Acetyl-CoA extraction was conducted using the method described earlier with a few modifications. Cells (150 ml) grown in TSB broth until mid-log phase were harvested by centrifugation at 8000 x g for 10 minutes, resuspended in a mixture of 10 mM sodium phosphate (pH 7.5), 10 mM $MgCl_2$ and 1 mM EDTA were extracted with ice-cold perchloric acid (0.5 M $HClO_4$) for 40 minutes on ice with intermittent vortexing. The suspension was centrifuged for 5 min at 2800 x g and approximately 2 to 5 ml of supernatant was neutralized with saturated $KHCO_3$, concentrated by

lyophilization to reduce the volume to 20% of the original (18). HPLC protocol for separation and quantification is described below.

HPLC conditions for separation of acetyl-CoA

The lyophilized extracts were finally dissolved in 500 µl of water. 50 µl. of supernatant was injected onto C_{18} reverse phase column (Agilent 5 HC-C18 (2) 250 X 4.6 mm). Samples were eluted at a flow rate of 0.8 ml/min. The buffers were 0.05 M potassium phosphate buffer (pH 4.7) (solution A) and pure methanol (solution B). The elution conditions were as follows: a gradient from 5% solution B to 25% solution B within 25 min, a gradient to 50% solution B within 8 min (33 min), and a gradient to pure solution B within 1 min (34 min). After this, the column was run isocratically with 5% solution B for 5 min (39 min). Acetyl-CoA from Sigma was served as standard (19).

2.6.23 Assay of *in vivo* role of chromomycin

The sub-inhibitory concentration of chromomycin (Sigma) used for *in vivo* studies was 315 $ng.ml^{-1}$ for JP1. Determination of minimum inhibitory concentration (MIC) of chromomycin is described above. Ascorbic acid was added at the final concentration of 200 $\mu g.ml^{-1}$. Each of the two compounds was added to the culture at the start of the growth. At 40-46 h and 96-120 h JP1 culture was withdrawn for plating, confocal microscopy and enzymatic assays.

2.6.24 Preparation of samples for RT-PCR for expression analysis of chromomycin biosynthesis genes:

The protocol is as described previously (1). Briefly, 10^8/ml of fresh spores were collected, pregerminated, and then inoculated in R2YE broth. Cultures were drawn at indicated time points (12 h, 17 h, 22 h, 27 h, 32 h, 38 h, 48 h and 60 h), were spun down and used for RNA extraction (RNA easy kit from Ambion) as described. Bacterial cells were collected at different time points i.e. at mid-log phase and stationary phase of incubation from broth and spun down at 4^oC. After a brief lysozyme treatment (0.2 to 0.5 mg/ml for 5 min. at 37 ^oC) total RNA was extracted by using phenol/guanidine thiocyanate mix Trizol reagent (Invitrogen) according to manufacturer's protocol. Quality and quantity was assured by visualizing RNA on agarose gel and acquiring absorbance at 260/280 ratio respectively. Residual DNA was removed by treating with DNAse (Ambion). PCR was performed using *hrdB*

primers to check removal of DNA contamination. Equal amount of RNA i.e. 2 µg was used from all samples for RT-PCR (Invitrogen kit). The cDNA thus prepared was directly used for expression studies (30 PCR cycles were performed) using *hrdB* as internal control. List of primer pairs used for the RT-PCR analysis are presented in Table 2.3.

Table 2.3 List of primer pairs used for RT-PCR analysis

Genes	Primers
pfk1 L	acctggtcaagatggtcgag
pfk1 R	accgtcttcagctccgtgac
pfk2 L	atgcgggtcggagtactgac
pfk2 R	aggtcgttgtcgatggtctt
pfk3 L	gcgtattggtgtcctcacct
pfk3 R	tgtcgttgtcgatggtcttc
Zwf L	gagtccttcaccgagtccag
Zwf R1	gagcatctcgtcggcttc
Zwf R2	gagcatctcgtccgcctc
cat1R	gttcgacctgaccaaggtgt
cat1L	ttctcctcctccgtcatcag
cat2L	ggctcaacctgaagatcctg
cat2R	gtcttgaagcccatcgactc
cat3 L	agtaccactggatgcccaag
cat3 R	ggtgtcgctgtaggagaagg
cat4 L	agatgtccgtgatgatgcag
cat4 R	gggtcgtcggtcatgttgta
sflD L	cgtacttcttcacccccatc
sflD R	gccgatatagctgtcctcca
sflRI L	Tatccgatgcacgaaaacct
sflRI R	Ctgtccacctcggtgaagtc
sflRII L	aaggtgtaccgcatcaccag
sflRII R	aacatcgcttcgtccaatg
hrdB L	ccggtcaaggactacctcaa
hrdB R	gtggcgtacgtggagaactt
thioR	aatggtacccagagagcgacgacttcc
thioL	aatggtaccatcactgacgaatcgaggt
cmmPL	aatctgcagcacgggaactgatccttgag
cmmpR	ttactgcagaggtgagaggcacgtagtcg
omtL	aatctgcagtacaccccgtatccgtcaat
omtR	ttactgcagggtctgaaacggctgaaaaa

2.6.25 Diamide and H_2O_2 sensitivity test

Equal number of spores (10^8/ml) of wild type was spread directly on surface of minimal agar medium containing glucose and that containing 1% of casamino acids. Different concentrations of each of diamide and H_2O_2 were placed in the wells created by cup borer. Zone of inhibition was examined after 36-48 hours of incubation of plates at 30°C.

2.6.26 *In vitro* antioxidant property of chromomycin

Tetracyclin hydrochloride, Chromomycin and ascorbic acid were obtained from Sigma.

DPPH assay : DPPH assay was performed as described in (20). 100 µl each of chromomycin, tetracyclin and ascorbic acid were added to 60 µM ethanolic solution of DPPH. Six concentrations were tested in triplicate. After mixing and incubating at 30°C for 30 minutes, readings were taken at 517 nm. The reaction results in change in color from deep-violet to light-yellow which is monitored spectrophotometrically at 517 nm. Ascorbic acid has been used as reference antioxidant in this assay.

The reduction of DPPH radical was expressed as percentage:

Scavenged DPPH (%) = $(1 - A_{test}/ A_{control}) \times 100$,

Where A_{test} is absorbance of a sample at a given concentration after 30 min reaction time and $A_{control}$ is the absorbance recorded for ethanol. IC_{50} value is defined as the concentration of sample that causes 50% loss of the DPPH radical.

FRAP assay : FRAP assay was performed as described by Benzie and Stain (21). FRAP Reagents included 300 mM acetate buffer, pH 3.6 with 16 ml glatial acetic acid per liter of buffer solution; 10 mM TPTZ (Sigma) in 40 mM HCl; 20 mM $FeCl_3.6H_2O$ working FRAP reagent was prepared by mixing 25 ml of acetate buffer, 2.5 ml TPTZ and 2.5 ml $FeCl_3.6H_2O$. 1 ml of FRAP reagent was added to 1 ml of different concentrations of chromomycin, tetracyclin and ascorbic acid were added. In this assay ferrictripyridyltriazine (Fe III TPTZ) complex is reduced to its ferrous form (blue colour) which can be monitored at 593 nm. The total reducing power of electron donating antioxidant in the reaction is related to change in colour.

FRAP value of Sample (µM) = (Change in absorbance of sample from 0 to 4 minute / Change in absorbance of standard from 0 to 4 minute) x FRAP value of standard (1000 µM). FRAP value of ascobic acid is 2.

Ferric ferrozine assay: The ferric-ferrozine complex solution was prepared as described by Apak *et al.*, (22). Different concentrations of chromomycin, tetracyclin and ascorbic acid as standard were used to make final volume of 0.5 ml in ethanol to which 1.5 ml of ferric ferrozine solution, 2 ml of buffer (sodium acetate, pH 5.5, 200 mM) and 0.5 ml of water was added to make final volume to 4.5 ml. After 30 min of incubation at room temperature absorbance was measured at 562 nm. Total antioxidant capacity is expressed as mM Trolox equivalent using 6.01 $x10^4$ as an apparent molar absorbtivity of trolox/l $mol^{-1}cm^{-1}$.

2.7 General methods

2.7.1 DNA ISOLATION

Plasmid isolation from *E. coli* by alkali lysis method (23)

Resuspend bacterial pellet, obtained from 10–30ml culture, in 1ml chilled solution I (GTE). After 5 minutes of 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 triple distilled water (TDW).

2.7.2 Introduction of DNA into cells

2.7.2.1 Electroporation in *E. coli*

Preparation of competent cells: Competent cells for electroporation were prepared as described by Sambrook *et. al.,* (23) 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 0.6 at 600 nm The cells were chilled on ice and

centrifuged at 4000 rpm for 10 minutes at 4ºC. The pellet was resuspended in 100ml of TDW, centrifuged at 4000 rpm for 10 min., followed by resuspension in 80ml TDW, 40ml TDW and 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 competent 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 37^0C for 45 min for expression of antibiotic resistance. The culture was then plated on appropriate antibiotic containing LB plates, incubated at 37°C for transformats to appear after 12 to 24 hours.

2.7.2.2 Transformation of *E. coli* using $CaCl_2$ (23)

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 minutes at 4°C. Supernatant was discarded and the pellet suspended in last drop. Add 0.1 M $MgCl_2$ (0.1 volume of culture volume) and keep the tube in ice for 10 minutes, spin for 5 min. at 5000 rpm at 4°C. Discard the supernatant and resuspend the pellet in 0.1 ml of calcium chloride (0.1 volume of culture volume) (chilled). Keep in ice for 10 minutes, spin for 5 min. at 5000 rpm at 4°C. Discard the supernatant; resuspend the pellet in last drop.

Incubate on ice for 30 minutes and add 5-7 µl of DNA mix the content by tapping the tube. Incubate further for 30 minutes in ice. Give heat shock treatment to cells at 37-42°C for 90 Sec, rapidly transfer to ice bath. Add LB and incubate for 45 min. at room temperature. After incubation period the tube was spin down for 5 minutes 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.

2.7.3 Transformation in *Streptomyces*

2.7.3.1 Preparation of protoplasts

Method- 1

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 till mid-log phase. Spin the culture and discard the supernatant, give the pallet 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 minuts, give the pallet 2 washes of P buffer and resuspend the pellet in last drop.

Method- 2

One ml of a frozen culture of *Streptomyces* was diluted into 9 ml of TSB broth and grown for 18hr aerobically at 29^{o}C. The culture was homogenized and 5 ml was transferred into 45 ml of fresh TSB broth supplemented with 0.8% glycine and grown for 16 hr 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 1 mg/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 R2YE plates. Incubate the plates at 30°C. After 14-20 h overlay with soft agar containing antibiotic. Score for resistant colonies after 3 days.

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

2 µl of 1 M NaOH added to 9 µl of DNA (1-5 µg), was mixed by tapping and incubated at 37°C for 10 min and rapidly chilled on ice. 2 µl of 1 M HC1 was added, and the contents were stored on ice till use.

2.7.3.2 Conjugation between *E. coli* and *Streptomyces*

The *E. coli* donor strain - S17.1 (containing RP4 derivative), was grown overnight at 33°C in Luria broth plus respective antibiotic, sub cultured 1:100 and grown further for 3 hrs at 33°C. The cells were paletted, washed twice with Luria broth and resuspended in 2 ml LB (donor culture). Spores of recipient *Streptomyces* were collected in 2XYT medium and were induced to germinate by heat shock at 50°C for 15 min. Equal volumes of the donor culture (*E. coli* cells 0.1 ml, 2 X 10^8 cells/ml) recipient (10^7 *Streptomyces* spores) were mixed, and 100µl was plated onto Soyabean Mannitol agar plates 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 antibiotic. Incubation at 30°C was continued for about a week to allow outgrowth of the exconjugants.

2.8 Genomic DNA library preparation

Partial digestions of genomic DNA were carried out for 3, 5, 7.5 min. time intervals at 37°C, using frequent cutter restriction enzyme *Sau3A1*. Reaction was stopped by immediately raising the temperature to 94°C. Electrophoresis of reaction product with Lambda *HindIII-EcoR1* marker was carried out in 0.8% agarose gel. The DNA partials were used for ligation with cloning vector. Ligation mixture was introduced in DH5α by electroporation. The transformants were directly screened on antibiotic to obtain a positive clone.

2.9 References

1. Kieser T (2000) *Practical streptomyces genetics* (John Innes Foundation).
2. Sambrook J, Fritsch EF, & Maniatis T (1989) *Molecular cloning: a laboratory manual* (Cold spring harbor laboratory press).
3. Zhao Y, Xiang S, Dai X, & Yang K (2013) A simplified diphenylamine colorimetric method for growth quantification. *Applied microbiology and biotechnology* 97(11):5069-5077.
4. Lowry OH, Rosebrough NJ, Farr AL, & Randall RJ (1951) Protein measurement with the Folin phenol reagent. *J biol Chem* 193(1):265-275.
5. Manteca A, Fernandez M, & Sanchez J (2005) A death round affecting a young compartmentalized mycelium precedes aerial mycelium dismantling in confluent surface cultures of Streptomyces antibioticus. *Microbiology* 151(Pt 11):3689-3697.

6. Decker LA (1977) Worthington enzyme manual.
7. Zou P, Borovok I, Ortiz de Orue Lucana D, Muller D, & Schrempf H (1999) The mycelium-associated Streptomyces reticuli catalase-peroxidase, its gene and regulation by FurS. *Microbiology* 145 (Pt 3):549-559.
8. Beauchamp C & Fridovich I (1971) Superoxide dismutase: improved assays and an assay applicable to acrylamide gels. *Anal Biochem* 44(1):276-287.
9. Borodina I, *et al.* (2008) Antibiotic overproduction in Streptomyces coelicolor A3 (2) mediated by phosphofructokinase deletion. *Journal of Biological Chemistry* 283(37):25186-25199.
10. Kennedy MC, Emptage MH, Dreyer JL, & Beinert H (1983) The role of iron in the activation-inactivation of aconitase. *J Biol Chem* 258(18):11098-11105.
11. Romanov VI, Hernandez-Lucas I, & Martinez-Romero E (1994) Carbon Metabolism Enzymes of Rhizobium tropici Cultures and Bacteroids. *Appl Environ Microbiol* 60(7):2339-2342.
12. Rui B, *et al.* (2010) A systematic investigation of Escherichia coli central carbon metabolism in response to superoxide stress. *BMC Syst Biol* 4:122.
13. MacKenzie ED, *et al.* (2007) Cell-permeating alpha-ketoglutarate derivatives alleviate pseudohypoxia in succinate dehydrogenase-deficient cells. *Mol Cell Biol* 27(9):3282-3289.
14. Seaver LC & Imlay JA (2001) Alkyl hydroperoxide reductase is the primary scavenger of endogenous hydrogen peroxide in Escherichia coli. *J Bacteriol* 183(24):7173-7181.
15. Singh R, Lemire J, Mailloux RJ, & Appanna VD (2008) A novel strategy involved in [corrected] anti-oxidative defense: the conversion of NADH into NADPH by a metabolic network. *PLoS One* 3(7):e2682.
16. Klaidman LK, Leung AC, & Adams JD, Jr. (1995) High-performance liquid chromatography analysis of oxidized and reduced pyridine dinucleotides in specific brain regions. *Anal Biochem* 228(2):312-317.
17. Price-Whelan A, Dietrich LE, & Newman DK (2007) Pyocyanin alters redox homeostasis and carbon flux through central metabolic pathways in Pseudomonas aeruginosa PA14. *J Bacteriol* 189(17):6372-6381.
18. Lesley JA & Waldburger CD (2003) Repression of Escherichia coli PhoP-PhoQ signaling by acetate reveals a regulatory role for acetyl coenzyme A. *J Bacteriol* 185(8):2563-2570.
19. Uchino K, Saito T, Gebauer B, & Jendrossek D (2007) Isolated poly(3-hydroxybutyrate) (PHB) granules are complex bacterial organelles catalyzing formation of PHB from acetyl coenzyme A (CoA) and degradation of PHB to acetyl-CoA. *J Bacteriol* 189(22):8250-8256.
20. Lertvorachon J, *et al.* (2005) 1,12-substituted tetracyclines as antioxidant agents. *Bioorg Med Chem* 13(15):4627-4637.
21. Benzie IF & Strain JJ (1996) The ferric reducing ability of plasma (FRAP) as a measure of "antioxidant power": the FRAP assay. *Anal Biochem* 239(1):70-76.
22. Berker KI, Güçlü K, Demirata B, & Apak R (2010) A novel antioxidant assay of ferric reducing capacity measurement using ferrozine as the colour forming complexation reagent. *Analytical Methods* 2(11):1770-1778.
23. Sambrook J, Russell DW, & Russell DW (2001) *Molecular cloning: a laboratory manual (3-volume set)* (Cold spring harbor laboratory press New York).