Chapter 7

Partial characterization of the chromomycin overproducer mutant - #OP

During the course of our study, an interesting and novel chromomycin overproducer mutant of *Streptomyces flavisclerotic*us was isolated which produced chromomycin ~176 fold more than the wild-type. Moreover, the overproducer mutant showed high level resistance to the aminoglycoside antibiotic apramycin unselected and exhibited some atypical features like profuse sporulation, increased growth rate on solid agar plate and extended viability. The present chapter describes the partial characterization of this chromomycin overproducer mutant (#OP) defending the merits of further studies.

7.1 Introduction

7.1.1 Effect of alteration of primary metabolite on secondary metabolism

The dedicated secondary metabolic biosynthetic pathway utilizes precursors and cofactors of primary metabolism (Table 7.1) and hence both pathways are intricately linked with each other. Thus any alteration in the primary metabolic pathway can cause alteration in the secondary metabolism. (Fig. 7.1).

Table 7.1. Precursors and cofactors required by different antibiotics

Antibiotics	Precursors required from metabolism	Cofactors required
Penicillin, Cephalosporin (β- lactam)	Acetyl-CoA, α-KG, Pyruvate, 3– phospho-glycerate, L-α-aminoadipic acid, valine and cysteine	NADPH
Anthracyclin, tetracyclin and actinorhodin (type II PKS)	Acetyl-CoA and Malonyl-CoA	NADPH
Rapamycin, Ascomycin, Nystatin, Rifampicin (type I PKS)	Acetyl-CoA, Butryl-CoA, propionyl-CoA, methylmanonyl-CoA	NADPH
Vancomycin, teicoplanin, dalbavancin (glycopeptides)	Phosphoenolpyruvate, erythrose 4-phosphate, Acetyl- CoA, sugars, lipids & amino acids	NADPH
Polymexin (polypeptide)	Oxaloacetate, pyruvate, Phosphoenolpyruvate, erythrose 4-phosphate, Acetyl-CoA,	NADPH

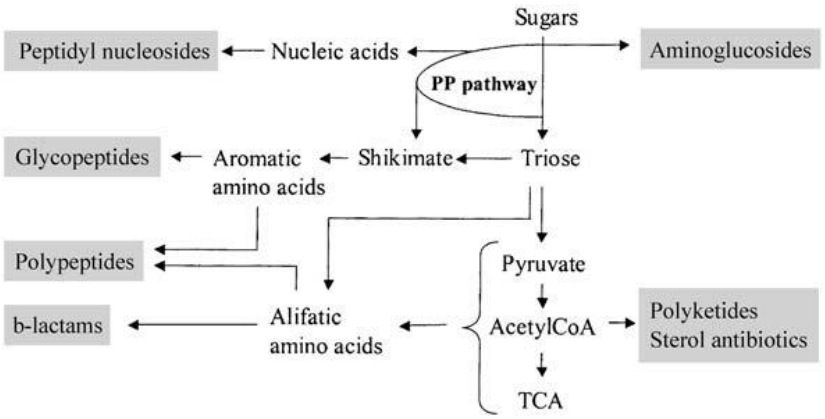

Figure 7.1 Overview of precursor requirement for secondary metabolism {Figure adopted from reference (1)}

Examples supporting aforementioned concept are - (i) Clavulanic acid (CA) requires arginine and glyceraldehyde 3-phosphate for clavulanic acid production by *Streptomyces clavuligerus.* Disruption of *gap1* encoding glyceraldehyde 3-phosphate led to improved CA production. In addition, arginine supplementation further increased the production of CA (2), (ii) overexpression of acetyl-CoA carboxylase (*acc*) in *S. coelicolor* led to improved actinorhodin production (3), (iii) Deletion of tricaboxylic acid cycle genes like citrate synthase (*citA*) or aconitase (*acoA*) in *S. coelicolor* resulted in organic acid overproduction, changes in secondary metabolite production and morphological differentiation (4, 5), (iv) In *S. lividans* polyphosphate kinase (*ppk*) gene inactivation led to activation of actinorhodin production which is usually silenced in this species (2, 6, 7) and (v) In *S. coelicolor*, the deletion of phosphofructokinase gene (*pfkA2*), encoding key enzyme of glycolysis led to improved production of antibiotics, actinorhodin and undecylprodigiosin with increased flux to pentose phosphate pathway (8).

It was observed earlier that methylenomycin production in *S. coelicolor* was supported by increased production of NADPH because of carbon flux favoring PPP pathway (9) suggesting that increased NADPH levels aids in enhancement of antibiotic synthesis by raising the Zwf (glucose 6-phosphate dehydrogenase) activity.

On the contrary, deletion of genes encoding glucose 6-phosphate dehydrogenase (*zwf1* and *zwf2*) in *S. lividans* led to antibiotics actinorhodin and undecylprodigiosin overproduction because of efficient glucose utilization by glycolysis, consistent with the observation that the deletion of the gene *devB* encoding 6-phosphogluconolactonase, the next step of PPP pathway, also resulted in increased antibiotic synthesis. Thus strain specific effects are seen on antibiotic production upon alteration of primary metabolism.

7.1.2 Antibiotic overproduction

As the production of 'antibiotics' is metabolically costly to the cell, in nature they are thought to have evolved to confer some selective advantage to the producing organism (10). However, from a biotechnological perspective, these amounts are far lower than those necessary for industrial-scale production. The producing organisms undergo significant strain improvement to yield the desired metabolite at an industrial scale.

Great deal of work is being carried out for the improvement in efficiency of antibiotic production by the producer strain, *Streptomyces*. The traditional convention for improvement of secondary metabolites is altering various physico-chemical factors. Random mutagenesis approach has been used in industry for strain improvement. Metabolic engineering is in demand to maximize product yields. It includes: alteration of metabolic flux, deregulation of specific biosynthetic pathway, inducing resistance to several antibiotics, overexpression of structural genes, genome shuffling, and expressing biosynthetic gene cluster in heterologous host (11, 12). These are labor and cost intensive approaches. Ribosome engineering is an innovative approach to increase antibiotic production. This approach involves engineering drug resistant mutations in rRNA or ribosomal proteins, several of them have been shown to cause oversynthesis of various antibiotics (13-15). As proposed by Ochi *et. al.*, (16) increased stability of the ribosome in ribosome targeting antibiotic resistant mutants is responsible for enhancement of antibiotic production in *Streptomyces* spp. Introduction of several drug resistant mutations targeting ribosomes has cumulative effect on antibiotic production. For example, octuple mutants of *S. coelicolor* resistant to antibiotics streptomycin, gentamycin, rifampicin, geneticin, paramomycin, fusidic

acid, lincomycin, and thiostrepton produce large amounts of the polyketide antibiotic - actinorhodin, 180-fold higher than the level produced by the wild type (16).

In the current chapter, isolation and partial characterization of a novel chromomycin overproducer mutant of *Streptomyces flavisclerotic*us, christened #OP, is described. The overproducer mutant is also resistant to the aminoglycoside antibiotic, apramycin unselected and cross-resistant to other aminoglycosides such as kanamycin, geneticin, gentamycin, and tobramycin but not to amikacin, parmomycin and streptomycin. #OP synthesizes chromomycin ~176 fold more than the wild-type (Fig. 7.2; 7.4, Table 7.2). Similarly, 70% of spontaneous apramycin resistant mutants of the wild type also overproduce chromomycin, implying the two phenotypes to be the result of a single mutation (Fig. 7.2). Moreover, atypical features like profuse sporulation, increased growth rate and viability are also observed for the mutant. This is in contrast to the finding that antibiotic resistant mutation is generally associated with compromised growth rate (15).

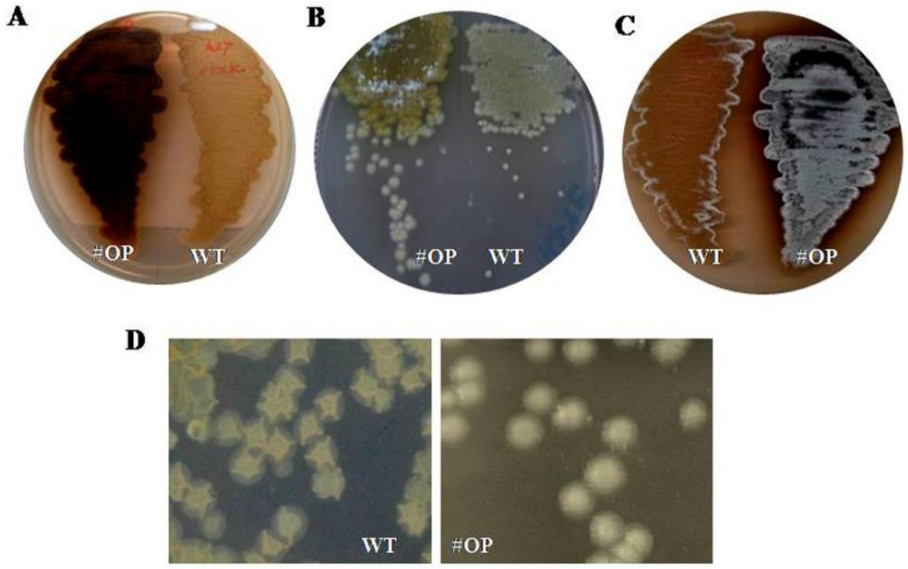

Figure 7.2 Phenotypes of chromomycin overproducer mutant - (A) chromomycin overproduction; (B) increased growth rate; (C) enhanced sporulation and (D) round colony morphology of #OP as compared to wrinkled colony morphology of the wild type on R2YE agar plates.

7.2 Results

7.2.1 Quantitation of growth rate of #OP and the wild type

Changes in growth rate, metabolism and morphology assisted the developmental program of the spore formation in *Streptomyces*. It is well recognized that the developmental life cycle of *Streptomyces* on solid media like R2YE starts with germination of spores leading to hyphae formation. Hyphae grow by tip extension and branch into a vegetative mycelium that grows across and deep down into the substrate where they form dense mycelium giving rise to the vegetative colony. *Streptomyces* colonies can withstand the nutrient starvation which occurs locally as they are non motile, it produces a second filamentous cell type, the aerial hyphae that grow up from the colony surface at the expense of lysis of substrate hyphae. The aerial hyphae further differentiate into spores (as described in chapter 1) (6).

The interesting features of #OP are round colony morphology and rapid growth in comparison to the wrinkled colony morphology and slow growth of the wild type on solid R2YE (Fig. 7.2). In order to assess the growth rate of #OP and the wild type, equal number of spores of each were inoculated in tryptone soy broth and the growth rate was monitored by drawing aliquots of culture at different time points and estimating the protein content at each time point. The result (Fig. 7.3) is interesting; the growth of #OP and WT is same all the way up to the stationary phase in the liquid broth which clearly does not account for the drastic difference observed between the WT and #OP mutant when grown on solid agar media (Fig. 7.2). Thus the growth on solid agar might be deceptive, as the shriveled growth front of the wild type colony is responsible for the apparent smaller size in comparison to the smooth colony morphology of #OP. Alternatively, comparative proteomic analysis of *Streptomyces ceolicolor* grown in solid and liquid media indicates complex differentiation process in liquid media as compared to solid media. The most remarkable protein abundance differences are seen in solid and liquid culture with the final stages of hyphae compartmentalization and spore formation (17). Transcriptomic analysis of *Streptomyces coelicolor* differentiation in solid sporulating cultures and liquid cultures indicates that the genes related to secondary metabolite biosynthesis

are up-regulated in liquid culture but not in solid cultures and the genes involved in final stages of hydrophobic cover/spore maturation are upregulated in solid but not in liquid cultures (18). Yague *et. al.*, (2014) illustrated that several non-characterized genes are differentially expressed in liquid and solid cultures which might be regulating metabolic and developmental differences between liquid and solid cultures (18). Early growth phase expression of differentiation genes including sporulation genes of #OP might also be the probable reason behind dissimilarity in the growth rate between WT and #OP observed on solid and liquid media. Comparative gene expression analysis of the wild type and #OP may provide insight into the differences observed.

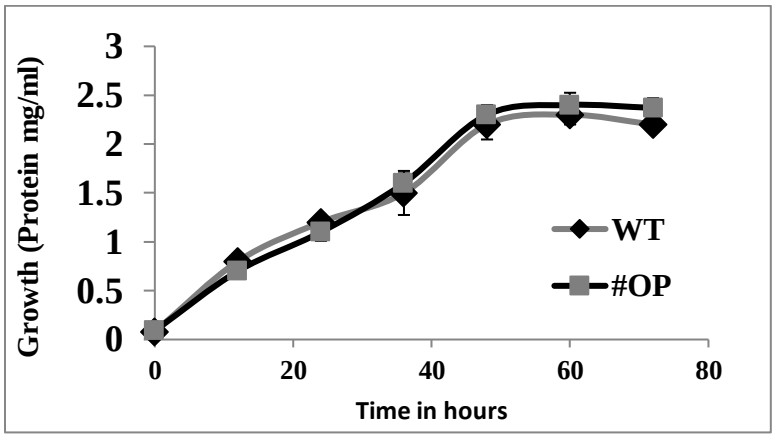

Figure 7.3 Growth rate of WT and #OP measured by protein estimation of cultures withdrawn at different time points from TSB media. The plot is representative of three independent experiments.

7.2.2 Quantitation of antibiotic production by #OP in comparison to wild type on different growth media

In order to quantitate amounts of chromomycin overproduced by #OP in comparison to the wild type, equal number of spores of both of #OP and WT were plated on different solid media- soyabean mannitol agar (SMA), R2YE Medium and TSB medium and the plates were incubated at 30^0C for 5-6 days. The chromomycin production in each medium was estimated by extracting chromomycin from equal number of plates in ethyl acetate as described in chapter 2 and the absorbance maxima of the extract at 405nm was measured; the concentration of chromomycin produced is

expressed in μg.ml^{-1}. It was found that #OP produces more chromomycin as compared to the wild type in the order: Soyabean Mannitol Agar > R2YE Medium > TSB (Table 7.2). Same result is reproduced on chromatography of the crude ethyl acetate extract on TLC silica plate (Fig. 7.4). It becomes clear from Table 7.2 that #OP produces ~176 fold more chromomycin in relation to WT.

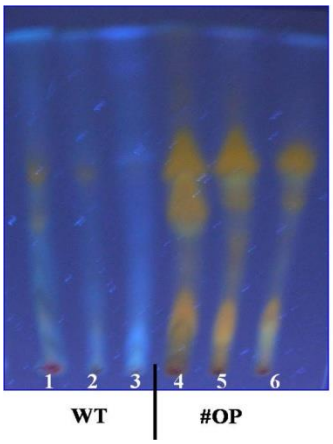

Figure 7.4 Representation of TLC plate developed for chromomycin extracted from WT and #OP grown on different media - 1 & 4 SM media, 2 & 5 R2YE media and 3 & 6 TSB media, exposed to UV light (365 nm). The experiment was repeated three independent times.

Table 7.2 Quantitation of chromomycin extracted from WT and #OP grown on different media

Strain	Concentration of chromomycin (μg.ml^{-1})		
	R2YE	SM	TSB
WT	0.65 ± 0.025	38 ± 2	11.8 ± 1.1
#OP	123.9 ± 8	147 ± 4	41.4 ± 1
Results are represented as mean ± SD of three independent experiments.			

7.2.3 Oxidative status of the overproducer mutant in comparison to the wild type

Consistent with the reparative functions of chromomycin in restoring viability to the non-producer mutant in stationary phase, and early gene expression of its

biosynthesis (chapter 6), the viability or longevity of #OP was found to be more as compared to the wild type. #OP was found to be viable for almost 12 to 15 days in liquid TSB whereas WT remains viable for only 7 to 8 days. The viability in stationary phase is a direct reflection of oxidative status even in prokaryotes (Chapter 4). For this reason, the levels of catalase and SOD in different phases of growth were measured. The results (Fig.7.5) demonstrate that in #OP the level of catalase is slightly on higher side but not to a significant extent which however continues to remain high for long in the stationary phase - the catalase activity doesn't decline till 7-10 days and is detectable even after 10 days. In contrast, in the case of the WT, the catalase activity was found to rise in the stationary phase (4-5 days) which declines with viability over the period (7-10 days). Considering that the catalase activity is a direct measure of viability, as is evident in our studies, it becomes clear why #OP is more viable than the WT. No major change in levels of SOD was found between WT and #OP. Thus appropriate management of oxidative stress in #OP assists its longevity is proved yet again in this case too.

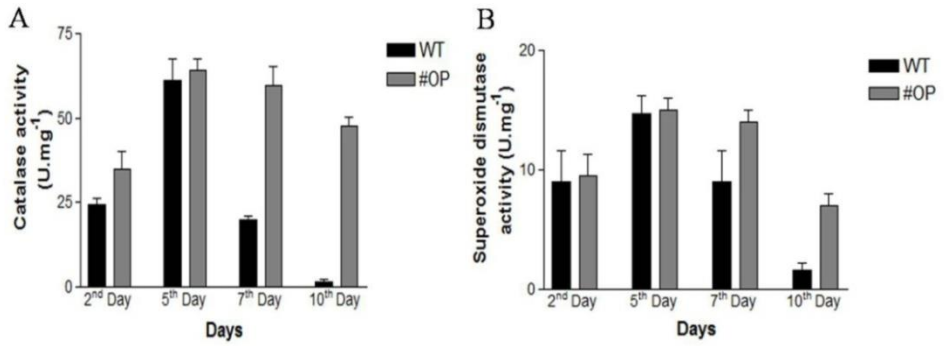

Figure 7.5 Status of antioxidant enzymes (A) catalase and (B) SOD in WT and #OP at different days of growth. The results are representative of three independent experiments; error bars indicate mean ± SD.

7.2.4 Metabolic alterations in #OP mutant: Glycolysis is upregulated in #OP

Glucose is metabolized primarily by glycolysis. The glucose flux through glycolysis can be monitored by assaying 6-phosphofructokinase (PFK) activity the regulatory, rate limiting and the first committed enzyme of glycolysis (19). In the case

of #OP, the ATP-PFK activity was found to be ~40% high probably due to upregulation of transcripts of *pfk2* and *pfk3* in #OP in relation to the WT (Fig. 7.6).

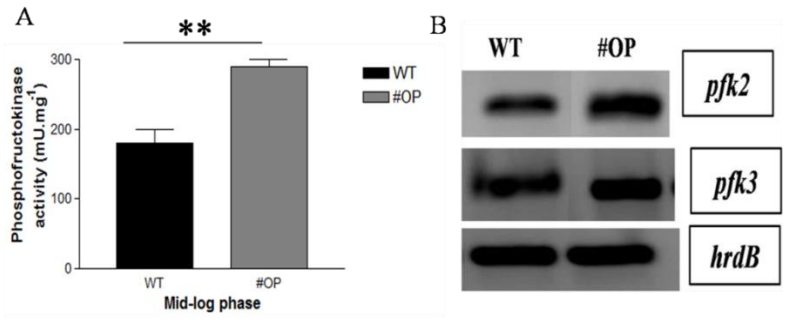

Figure 7.6 Glycolysis flux in #OP is measured by PFK activity and expression (A) ATP-PFK activity measured in mid-log phase of WT and #OP; (B) expression of *pfk* genes in mid-log phase of both the wild type and #OP. The results are representative of three independent experiments; error bars indicate mean ± SD. Statistically significant differences between strains at each time point were assessed Student's t-test:**$p < 0.01$.

7.2.5 NADPH production is limited in #OP

#OP and WT were exposed to diamide (0.5M) which oxidizes sulfide groups in proteins and thiols causing formation of toxic disulfide bridges. This oxidative stress is neutralized by thioredoxin reductase requiring NADPH as electron donor. The more NADPH the cells can generate, the higher the supply for thioredoxin reductase, and as a consequence, more resistant is the strain to diamide. To quell oxidative stress there is increased flux through PPP for enhanced production of NADPH (8). Higher sensitivity to diamide is indicative of low NADPH levels. The result in Fig. 7.7 indicates that #OP is highly sensitive to diamide than WT is (Table 7.3). Accordingly, the NADPH levels in #OP are ~2- to 3- fold less in comparison to the wild type (Table 7.3; Fig. 7.8). The above two results suggest a possible metabolic reconfiguration in #OP which needs to be examined further.

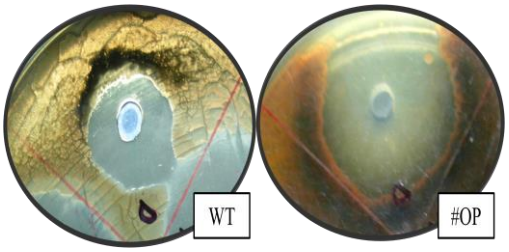

Figure 7.7 Sensitivity of WT and #OP to 0.5 M diamide on R2YE agar plates.

Table 7.3 The zones of inhibition of growth of WT and #OP formed by 0.5 M diamide

Strain	Diamide Sensitivity	
	Diameter in (mm)	Surface area (mm^2)
WT	30 ± 0.28	706.85
#OP	40 ± 0.38	1256.63
Results are represented as mean ± SD of three independent experiments.		

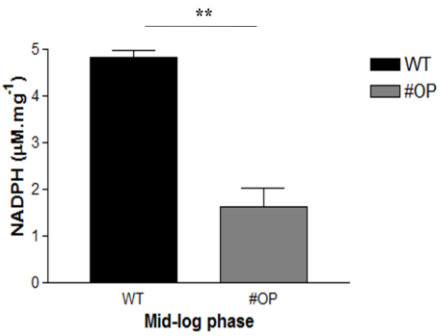

Figure 7.8 Intracellular levels of NADPH as measured by enzymatic cycling method in WT and #OP. The results are representative of three independent experiments; error bars indicate mean ± SD. Statistically significant differences between strains at each time point were assessed Student's t-test:**$p < 0.01$.

7.2.6 Modification of apramycin by *aac3 (IV)* gene encoding the aminoglycoside acetyltransferase does not result in chromomycin overproduction

Apramycin is an unusual aminoglycoside antibiotic with potent broad-spectrum activity. The mode of action of aminoglycoside antibiotics is that they impair bacterial protein synthesis by binding to the A (acceptor) site of the 30S prokaryotic ribosome. The most common aminoglycoside resistance is caused by enzymatic modifications leading to decreased affinity of the drug for the target. The 1 N-acetylation of amino groups on aminoglycoside is carried out by aminoglycoside acetyltransferases employing acetyl-CoA as the donor. The aminoglycoside acetyltransferase AAC-(3) family modifies 3-amino group of the deoxystreptamine ring. The family includes 4 types I-IV, which is based on pattern of aminoglycoside resistance they confer. The AAC(3)-I and –(II) modify gentamycin group of aminoglycosides. AAC(3)-(III) enzymes causes acetylation of antibiotics such as gentamycin, tobramycin and neomycin. The AAC(3)-IV class of N-acetyltransferases having broad substrate specificity out of all AAC(3) enzymes, they acetylate atypical aminoglycoside – apramycin (20). AAC(3)-IV is a plasmid encoded aminoglycoside acetyltrasnsferase in *E. coli*, *C. jujeni* and *P. stutzeri* (21, 22).

AAC(3)-IV is widely used as an antibiotic resistance marker in routine cloning and genetic manipulations in *Streptomyces*. In order to test the possibility that the apramycin resistance in #OP mutant is due to *aac3(IV)* gene, the integrating plasmid of *Streptomyces* pSET152 harboring *aac3 (IV)* gene was transferred to the wild type by conjugation. The ex-conjugants purified on agar plates supplemented with apramycin didn't show chromomycin overproduction ruling out the cause of apramycin resistance in #OP to be due to *aac3 (IV)* gene (Fig. 7.9). The cross resistance profile of #OP was same as that of WT (pSET152) (Table 7.4). Though the resistance profile apparently coincides that of acetyltransferase gene *aac(3)IV* of plasmid pSET152, acetylation of the antibiotic apramycin by *aac3(IV)* encoded enzyme is unlikely to be the reason of resistance to apramycin in #OP as explained earlier. The possibility of modification of the antibiotic at other positions cannot be ruled out.

Figure 7.9 The ex-conjugants (EX-2, -3, -4 and -5) of pSET152 plasmid integrated in the chromomsome of the wild type were resistant to apramycin (due to *aac(3)IV* gene) but didn't overproduce chromomycin.

Table 7.4 Resistance profile for different antibiotics for WT, #OP and pSET WT

Antibiotics	Concentration (µg)	WT	WT (pSET152)	#OP
Zones of inhibition (cm)				
Gentamicin	10	2.6	R	R
Geniticin	10	1.5	R	R
Kanamycin	10	1.2	R	1.5
Tobramycin	10	2.7	R	R
Amikamicin	10	2.9	4	3
Apramycin	10	2.5	R	R (200µg)
Hygromycin	500	2.75	1.5	1.5
Neomycin	10	2.2	R	1.5
Paromomycin	10	1.5	1.5	1.5
R- resistance				

In order to define the mechanism responsible for apramycin resistance and chromomycin overproduction in #OP, cloning of apramycin resistance gene from genomic library of #OP was attempted. Following is the brief explanation of the cloning strategy used.

7.2.7 Cloning of apramycin resistance mutation

Genomic DNA library construction is prerequisite to clone a targeted DNA. Library construction is referred to as creating clones carrying DNA fragments

representing complete genomic DNA of the organism of study. Appropriate vector selection is very important consideration as the gene fragments used in the library is often large. The choice of cloning vector, in turn, determines the method to deliver insert carrying DNA in the host. Most of the vectors used for genetic manipulations in *Streptomyces* have apramycin resistance as selectable marker, which cannot be used for library preparation given the selection of the chromomycin overproduction phenotype of #OP is based on the selection for apramycin resistant phenotype . pWHM3, procured from Prof G.P. van Wezel, Leiden University, The Netherlands, was selected as cloning vector because of reasons such as high copy number, small size (7.2 kb), thiostreptone gene resistance marker as the selectable marker and being the shuttle vector for both *E. coli* and *Streptomyces.* In order to make pWHM3 conjugable for transfer from *E. coli* to *Streptomyces, oriT* (0.8 Kb fragment required for RP4 mediated mobilization of plasmid from *E.coli* to *Streptomyces*) from plasmid pSET152 was cloned at an unique *Pst*I site of pWHM3 (Fig. 7.10).The resultant recombinant plasmid pWHM3–*oriT* was then confirmed by restriction digestion and was used for library preparation.

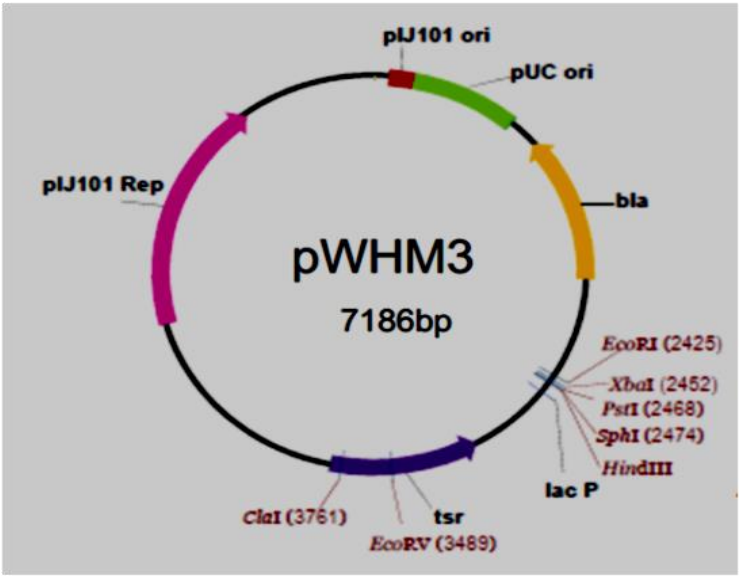

Figure 7.10 Vector map of pWHM3

7.2.7.1 Genomic DNA library preparation of #OP

Briefly, the genomic DNA was isolated from #OP and was checked for its purity by absorbance (OD) at 260nm/280nm and digested with *Sau3A*I (a frequent cutter) enzyme to obtain DNA fragments in size range of 2-5kb. The partial DNA fragments were ligated with *BamH*I digested pWHM3-*oriT* vector and transformed in *E.coli* (DH5α). Genomic library pool of around 50,000 transformants was obtained. In order to determine number of clones having insert, plasmid isolation was done from 10 colonies and *Pvu*II digestion was carried out in order to measure insert release and its size. The digestion pattern by *Pvu*II indicates that out of 10 colonies 2 clones have inserts of approx. 1.5 kb. It results in representation of 15 Mb of genomic DNA (1.5 x 10,000 = 15,000 kb). The genomic library pool was further transformed into the *E. coli* strain S17.1 (for intergeneric conjugation between *E.coli* and *S. lividans)* and in *E. coli* host ET12567 (a nonmethylating donor, for transformation in *S. flavisclerotícus* as it restricts methylated DNA). Apramycin resistance selection is carried out along with thiostreptone selection to clone apramycin resistant gene from #OP in the heterologous hosts of *Streptomyces* strains. After several transformation and conjugation attempts in *Streptomyces*, transformants resistant to both apramycin and thiostrepton were not obtained however single selection for thiostrepton resistance in *S. lividans* produced several thousand transformants in intergenus conjugation with *E coli* S17.1. Probable reason for this failure is explained below. Schematic representation of genomic library preparation is shown in Fig. 7.11.

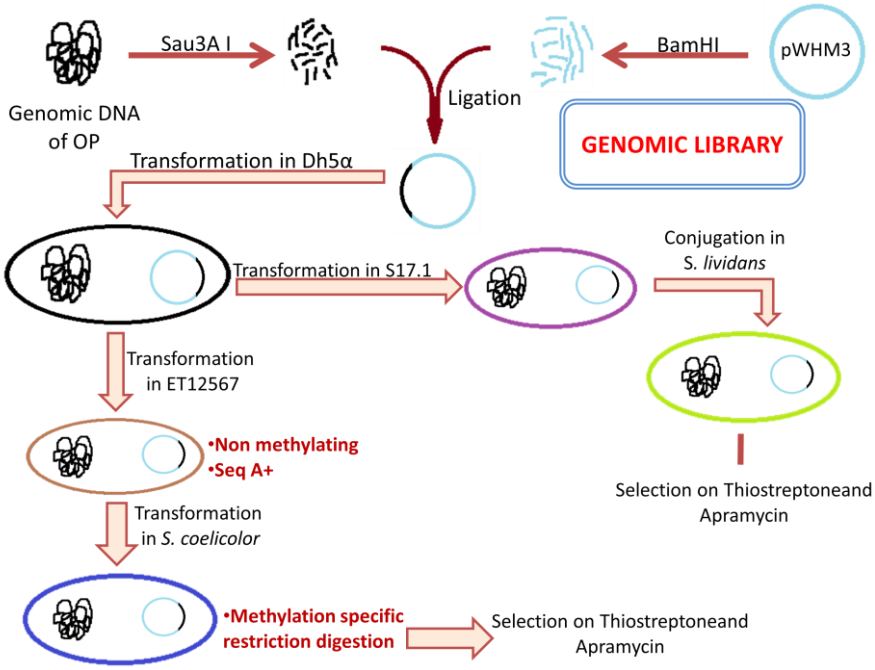

Figure 7.11 Schematic representation of genomic DNA library preparation of #OP

7.2.8 Attempt to uncover nature of apramycin resistance mutation

Ribosomal protein mutation or rRNA methyl transferase activity can reasonably explain improved growth of #OP on R2YE and one step resistance to apramycin and chromomycin overproduction in #OP as the mutant ribosome may be affected for translation efficiency/fidelity. Apramycin inhibits translocation of ribosomes by interacting with S12 protein and rRNA (T40 residue) (23). The ribosomal protein encoding genes' cloning can be difficult because of the antibiotic resistant mutant gene being recessive to antibiotic sensitive the wild type allele. Therefore, in order to rationalize the mutation in #OP is in a ribosomal protein encoding gene, following experimentation was conducted in which other ribosome acting drug resistant mutants of #OP were generated and their effects on overproducer phenotype were examined.

7.2.8.1 Generating other ribosome acting drug resistant mutants of #OP and their effect on the overproducer phenotype

Ribosome is a highly conserved RNA-protein complex which synthesizes proteins (24). Antibiotics frequently target the bacterial ribosome and its associated

translation factors to highly conserved functional sites. The antibiotic resistance mutations are generally found in these components (24). Given that ribosomes have multiple antibiotic target sites, it is possible that mutations that confer resistance to one antibiotic can cause increased sensitivity to other. In unpredictable ways, such ribosomal mutations potentially interact with one another which include the phenotypic suppression of one mutation by another. These phenotypic interactions can provide evidence of long-range functional interactions throughout the ribosome and its functional complexes and potentially give insights into antibiotic resistance mechanisms.

It is well recognized that introduction of streptomycin resistance mutation (*rpsL,* ribosome protein S12) in *S. ceolicolor A3(2)* and several other *Streptomyces* strains results in antibiotic overproduction (25). Combination of certain drug resistance mutations can further increase antibiotic production (14). Notably, though #OP is partially resistant to several aminoglycosides (Table 7.4) it is highly sensitive to streptomycin, paramomycin and amikacin. High level one step streptomycin resistance mutation maps to *rpsL*. In order to probe if the apamycin resistance mutation defines ribosomal component, streptomycin resistant mutants of #OP were generated at 25-30µg /ml concentration of streptomycin. The phenotypes of streptomycin resistant mutants of the wild type and #OP were examined. Unexpectedly, the Strep^r mutants of the wild type show severe loss of chromomycin production (Fig. 7.12; 7.14) which is clearly contrary to the results obtained so far in other species of *Streptomyces* where *rpsL* mutation leads to antibiotic overproduction (15). Similarly, in the case of #OP, streptomycin resistant mutation led to complete loss of apramycin resistance and also chromomycin overproduction (Fig. 7.13; 7.14), reinforcing the fact that the two mutations viz., apramycin resistance and chromomycin oversynthesis are the result of a single mutation. The results were corroborated by extracting chromomycin from equal number of plates of each of WT, #OP, Strep^r mutants of WT & #OP. The chromomycin extracted from each strain was separated on TLC plate and visualized under UV (365 nm). Additionally, concentration of chromomycin was determined by measuring absorbance at 405nm. The results, Fig. 7.14 and 7.15 reinforced that Strep^r mutants of the wild type

produced very little chromomycin and that of #OP lost chromomycin overproduction phenotype. This result is significant and indicates a negative interaction of streptomycin resistance mutation with mutational target for apramycin resistance. In order to prove the hypothesis that the apramycin resistance mutation defines ribosomal target, spontaneous mutants resistant to other ribosome acting drugs such as amikacin (acts on 16s rRNA and 30S ribosome), paramomycin (acts on16S rRNA and 30S ribosome) and erythromycin (acts on 23S rRNA) were generated. The phenotypes of the resistant mutants generated are as tabulated in Table 7.5. Although the implication of the findings are presently unclear, the genetic suppression of the apramycin resistant and chromomycin overproduction phenotypes of #OP is limited to streptomycin and amikacin resistant mutations each paramomycin and erythromycin resistance each did not alter #OP phenotypes (Table 7.5).

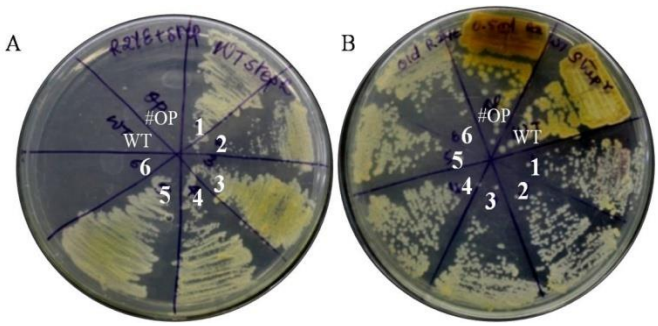

Figure 7.12 Phenotypes of streptomycin resistant mutants of the wild type (A) Strep^r mutants of the wild type (1-5) grown on R2YE containing streptomycin plates with WT and #OP as negative control (B) Reduced chromomycin production by Strep^r mutants of the wild type (1 to 5) on R2YE plates as compared to the Wild type and #OP.

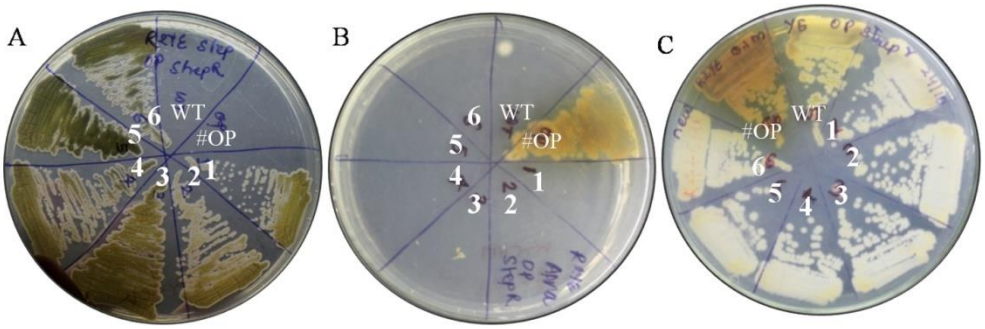

Figure 7.13 Phenotypes of streptomycin resistant mutants of #OP (A) Strep^r mutants of #OP (1 to 6) on R2YE with streptomycin (25 µg/ml) where the wild type and #OP are sensitive; (B) Strep^r mutants of #OP (1 to 6) on R2YE with apramycin (20 µg/ml) with the wild type as negative control and #OP as

positive control; (C) Severe reduction in chromomycin production in Strep^r mutants of #OP (1 to 6) in comparison to WT and #OP.

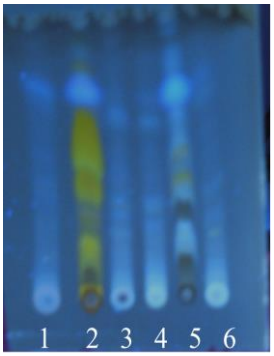

Figure 7.14 TLC of ethyl acetate residue of chromomycin extracted from indicated strains visualized under UV light (365 nm) (1) OP Strep^r mutant 1; (2) #OP; (3) OP Strep^r mutant 2; (4) WT Strep^r mutant 1; (5) WT; (6) WT Strep^r mutant 2

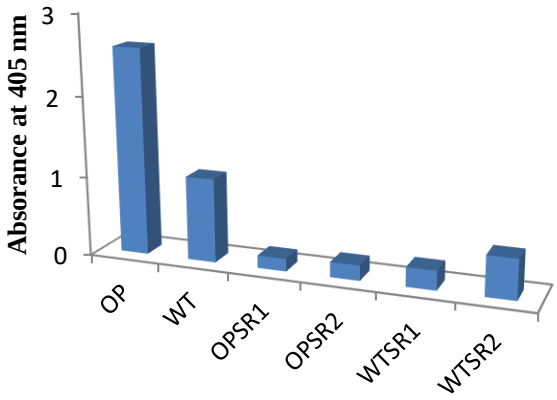

Figure 7.15 Absorbance of chromomycin at 405nm extracted from OP, WT, streptomycin resistant mutants of #OP - 1 and 2, Streptomycin resistant mutants of the wild type - 1 and 2

Table. 7.5 Phenotypes of various ribosome acting antibiotic resistant mutants of #OP and WT

Mutants	Apramycin resistance	Chromomycin overproduction
WT	S	++
#OP	R	++++
WT Strep^R	S	+
#OP Strep^R	S	+
WT Ami^R	S	+
#OP Ami^R	S	+
#OP Par^R	R	++++
#OP Ery^R	R	++++

7.2.8.2 Whole Genome sequencing of #OP

In order to characterize and evaluate genetic engineering potential of the mutation causing chromomycin overproduction in #OP, *de novo* whole genome sequencing of #OP has been attempted using Illumina NextSeq with Bionivid. The draft genome sequence has been submitted to NCBI with accession number MAZZ00000000. The S12 protein sequence obtained after whole genome sequencing was matched with S12 protein of other nearest *Streptomyces* spp. like *S. avermitilis.* It was observed that the sequence for ribosomal protein S12 was identical to the sequence of other organisms including the model organism *S. coelicolor*. Thus genetic mutation in S12 is not involved in the mechanism for apramycin resistance. The possibility of posttranslational modification in ribosomal proteins leading to apramycin resistance can be considered. Similarly, the sequence of the *gidB* gene encoding 16S rRNA methyl transferase, whose loss of function has been shown to cause low level streptomycin resistance (26), was found to be unaltered in the #OP.

7.2.9 Assessing the prospect of apramycin resistance mutation for overproduction of important metabolites: construction of chromomycin non-producer mutant as a heterologous host

This chapter also addresses if the #OP mutant of *S. flavisclerotícus* can be developed as a heterologous host for biotechnological application. It is necessary to determine if the apramycin resistance mutation in #OP can obviate the requirement of chromomycin for its growth, and viability as in the case of the non-producer mutant JP1 (Chapter 3 & 4).

The chromomycin non-producer mutant of #OP was constructed that was disrupted for glucose 4,6-dehydratase (*sflE*, gene involved in deoxysugar biosynthesis) The plasmid used for gene disruption (pSET152Δ*Pst*) is a derivative of pSET152 constructed by *Pst*I digestion and intramolecular ligation of pSET152 DNA which removes its genome integration function. ~ 8 kb PKS DNA containing highly conserved polyketide condensing functions encoded by promoter distal *sflK* and *sflP* in an operon organization with promoter proximal *sflD* and *sflE* (Fig.7.15) was cloned at the *Eco*RI site of the pSETΔ*Pst* plasmid to generate pSETΔ*Pst*-8kbPKS gene disruption vector. The disruption of gene for deoxysugar biosynthesis (*sflE*) was carried out by introducing the 'thiostreptone resistance gene casette' at the unique restriction site for the enzyme *Kpn*I in *sflE* (Fig.7.15) in the plasmid (pSETΔ*Pst*-8kbPKS). The insertion of thiostrepton resistance cassette not only disrupts the *sflE* gene but may also exert polar effect on the expression of promoter distal *sflK* and *sflP* genes unless an internal promoter allows for their expression (Fig.7.15). Importantly, the *sflE*::Tsr^r mutant gene on the plasmid is flanked by an equal amount PKS DNA for an unbiased homologous recombination between the suicide plasmid-born mutant PKS DNA with the corresponding DNA in the host chromosome. The plasmid DNA was propagated through the non methylating *E coli* host ET12567, rendered single stranded (as described in chapter 2) and transformed into WT and #OP mutant. Transformants were selected for thiostreptone resistance. Significantly, about 50% of a total of 100 transformants of WT were thiostreptone resistant and apramycin sensitive indicating their generation by a double cross over recombination (results described in chapter 3). The *sflE* mutants of the wild type showed severe growth

defect as rendered pure by successive rounds of purification for lack of chromomycin production. Since the transformants of #OP would be both thiostrepton- (selection marker) and apramycin resistant (due to mutation in #OP), there is no easy way to discriminate a single crossover integrant from double crossover mutant. Two thiostrepton resistant colonies were purified extensively by successive subculture of the spores and screened for colonies with diminished diffusion of yellow color (Fig.7.16). The chromomycin non-producer mutant was assessed for chromomycin production by measuring absorbance at 405nm of the chromomycin extracted from each *sflE* mutants. The results (Fig. 7.17) reinforced the conclusion that nonproduction of chromomycin doesn't affect apramycin resistance and other phenotypes of #OP such as profuse sporulation and robust colony morphology. Thus, it is clear that apramycin resistance mutation can obviate/suppress/overcome the requirement of chromomycin for viability and other phenotypes. Since there is dearth of antibiotic resistance marker genes in the case of *Streptomyces* (apramycin-thiostrepton-, hygromycin-, gentamycin-, kanamycin resistance are more commonly available on plasmids) and that #OP is cross resistant to several aminoglycosides such as kanamycin, hygromycin gentamycin etc., (Table 7.4) a markerless 'clean' mutation of chromomycin genes (preferably deletion of all the cluster genes) is required to be constructed for testing the expression potential of chromomycin non-producer mutant of #OP.

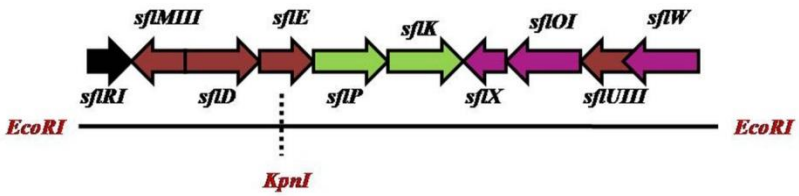

Figure 7.15 Genetic map of ~8 kb PKS DNA cloned at the *Eco*RI site of the *E. coli-Streptomyces* suicide vector pSETΔ*Pst* generating gene disruption vector pSETΔ*Pst* 8KbPKS. The unique *Kpn*I site in the *sflE* gene is used for cloning the thiostrepton resistance gene cassette for construction of chromosomal *sflE*::*tsr*^{R} knockout mutation.

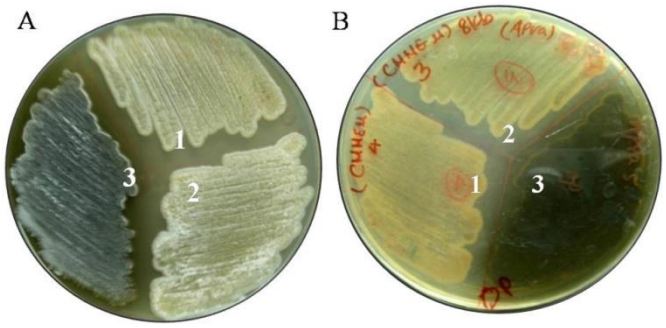

Fig. 7.16 Insertion mutants disrupted for *sflE* of #OP (gene involved in deoxysugar biosynthesis of chromomycin); 1 and 2 are *sflE::tsr* mutants of #OP and 3 is #OP. (A) Front and (B) back view of R2YE plates.

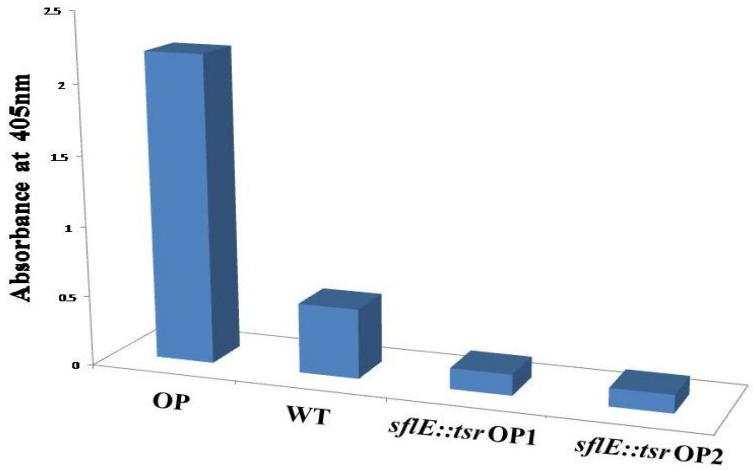

Figure 7.17 Absorbance of chromomycin at 405nm extracted from #OP, Wild type, *sflE* insertion mutants of #OP: *sflE::tsr*OP1 and *sflE::tsr*OP2

7.3 Discussion

The present chapter describes isolation and preliminary characterization of novel apramycin resistant mutation (in #OP) leading to chromomycin overproduction (~176 fold) in *Streptomyces flavisclerotic*us. The overproducer mutant is also cross-resistant to other aminoglycosides such as kanamycin, geneticin, gentamycin, hygromycin and tobramycin but not to streptomycin, amikacin, paramomycin and neomycin. The following evidence reaffirm the conclusion that the phenotypes of chromomycin oversynthesis and apramycin resistance is the result of single mutation – (i) 70% of spontaneous apramycin resistant mutants of the wild type overproduce

chromomycin; (ii) Independently isolated mutants which overproduced chromomycin were also apramycin resistant; (iii) Furthermore, the streptomycin and amikacin resistance mutation each suppressed both apramycin resistance and chromomycin overproduction phenotype of #OP. The atypical features such as apparent enhanced growth on solid agar media and viability are also characteristic of the mutant. The growth rate of #OP and WT was similar when measured in liquid medium (tryptone soya broth). The apparent enhanced growth rate of the mutant might be due to precocious early expression of differentiation and development genes including those of sporulation. Other possible reason could also be the differential expression of genes between the wild type and the pleiotropic mutant #OP on solid and liquid media as observed in other *Streptomyces* spp (17, 18).

The phenotype of extended viability of #OP could be related to its ability to manage oxidative stress by upregulating antioxidant enzymes in stationary phase i.e. 7 days -15 days. The viability in stationary phase is a direct reflection of better management of oxidative status in prokaryotes is reiteratively proved in these studies.

Indications of metabolic readjustments in #OP involved increase in the PFK activity providing the starter substrate acetyl-CoA, and reduced levels of NADPH probably implying an enhanced utilization of NADPH for increased levels of chromomycin synthesis (Fig. 7.6; 7.7; 7.8; Table 7.3). A thorough investigation of levels of some of the glycolytic, PPP pathway and Kreb cycle enzymes and their products is required for an understanding of the relevance of the altered levels of primary metabolic reactions supporting the secondary metabolite, chromomycin synthesis. However, these metabolic alterations are inadequate to overcome the inherent preference of #OP for amino acids as carbon source, as the growth of #OP and the wild type is same on minimal glucose media. Thus the additional amounts of acetyl-CoA for chromomycin overproduction might be generated by catabolism of amino acids into TCA cycle intermediates and thereby fueling gluconeogensis.

Chapter 7

Speculating on the mechanism of the apramycin resistance

Aminoglycosides (AGs) are natural antibiotics produced by soil dwelling *Actinobacteria*. They are structurally diverse consisting of two or more amino modified sugars linked to an aminocyclitol core. It binds to aminoacyl-tRNA recognition site (called as A-site) of 16S rRNA which constitutes ribosomal subunit 30S. Thus, polypeptide synthesis is halted and subsequently death occurs. Its mode of action also includes corruption of genetic code (27). These protein synthesis inhibitors also engender pleiotropic translational effects such as increase in misreading rates, inhibits tRNA-mRNA translocation and ribosome recycling (28, 29). In detail, these aminoglycosides bind to an asymmetric internal loop within helix 44 (H44) of small subunit 16S rRNA at the A-site of ribosome (30). This binding in turn affects tRNA selection and translocation catalysed by elongation factor G (EF-G) (28, 31). The AGs also targets 23S rRNA at helix 69 (H69) (32). When translation process is completed, H44 of 16S rRNA, H69 of 23S rRNA, EF-G and ribosome recycling factor interacts to form a complex to separate the two ribosomal subunits and to recycle the ribosomes (33, 34). Thus AG binding to these helices of either 16S or 23S rRNA hinders ribosome recycling and release of ribosomal subunits (33, 34). The mechanisms of bacterial resistance to aminoglycosides are diverse (Fig. 7.18). They include: (i) Enzymatic modification and inactivation of aminolgycosides – It is the most common resistance mechanism among Gram positive and negative bacteria, mediated by aminoglycoside modifying enzymes such as aminoglycoside acetyltransferase, nucleotidyltrasnferase or by phosphotransferase (35, 36); (ii) increased efflux which exclude AGs out of the cell; (iii) Decreased permeability via lipid modifications in cell wall resulting in repulsion of entry of AGs inside the cell; (iv) modification of 30S ribosomal subunit where either mutations occur at ribosomal targets (such as mutations in ribosomal protein and 16S rRNA) or by posttranscriptional modification, for instance, modification of ribosomal RNA by ribosomal RNA methyltransferases.

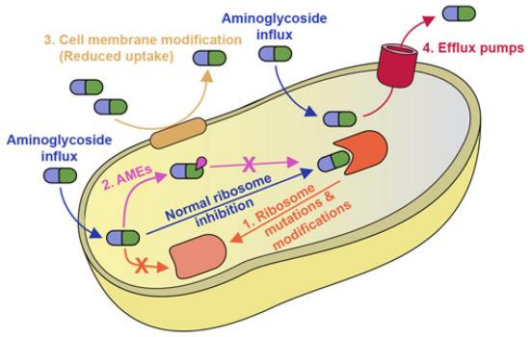

Figure 7.18 Mechanisms of resistance to aminoglycosides [Image source reference (37)]

Apart from aforementioned classical resistance mechanisms many more uncommon modes of resistance mechanisms to aminoglycosides have emerged in recent studies. For instance in *Salmonalla enterica* spp. (clinically isolated species) tobramycin resistance was attributed to activation of silent acetyltransferase due to molecular rearrangement resulting in a transcriptional fusion. The distribution of the gene and the environment suggested its putative role in sugar metabolism (38). The opportunistic pathogen *Pseudomonas aeruginosa* confers aminoglycoside resistance by diverse and remarkable mechanisms such as growing biofilms, conversion to mucoidy forms or by emergence of persister cells. Interestingly, recent advances suggest that resistance to AGs is associated with altered morphology of cells. A novel tobramycin resistant *P. aeruginosa* exhibited small colony morphology which was an outcome of combinatorial effects of three two-component systems (39). According to latest study, AG resistance can be acquired by a new mechanism in which mutation in elongation factor EF-G1A led to aminoglycosides – gentamycin, tobramycin and amikacin resistance (40). One of the study suggests that AG sensing riboswitch (non-coding m-RNAs which bind to small molecules and cofactors in order to regulate expression of biosynthetic genes) can control expression of aminoglycoside acetyl transferase gene (*aac*) or adenyl transferase (*aad*), by being part of the leader RNAs of *aac/aad*. The leader RNAs can bind to aminoglycosides which in turn causes structural changes in RNA and thereby inducing expression of *aac/aad* genes (41).

Number of studies on derivation of aminoglycoside resistance mechanisms suggests that they originated from both the producing organism and from mutations in

normal cellular genes (42, 43). High level expression of normally silent cellular genes can lead to aminoglycoside resistance (44). In certain cases the aminoglycoside resistance genes are derived from housekeeping genes of similar function. As proposed by Pipersberg (43) the aminoglycoside modifying enzymes are proposed to be involved in metabolism of low molecular weight compounds and enzymes of primary metabolism such as macromolecular synthesis, transport regulation and morphogenesis. They also showed that there is distant relationship between phosphotransferase and serine/threonine and tyrosine kinases (43, 45, 46). There is structural and functional similarity between adenyltransferase and DNA polymerase β indicating possible originality of adenyltransferases. The acetyltransferase AAC(3)-III of *Micobacterium fortuitium* was also found to play role in synthesis of TCA cycle intermediates (47), it can convert oxaloacetate to citrate and it can also acetylate ribosomal protein (as acetyltransferases were found to be similar to RIM proteins which acetylates ribosomal proteins (43)). Most probable reason for such multiple activities is structural similarity between oxaloacetate, ribosomal proteins and the aminoglycosides. Thus aminoglycoside modifying enzymes carry out modification of substrates resembling aminoglycosides. In bacteria, the polymers of amino sugars such as N-acetylglucoseamine and N-acetylmuramic acid are possible target of aminoglycoside modifying enzymes because of the presence of acetyl group. It is proposed that the inherent aminoglycoside acetyltransferase might have housekeeping role in peptidoglycan or LPS metabolism (48). The altered expression levels of these housekeeping aminoglycoside acetyltransferase genes might result in significant aminoglycoside resistance (48). Regulatory mutations or gene amplification result in increased expression of these acetyltransferases leading to high levels of aminoglycoside resistance. The role of aminoglycoside transferase in peptidoglycan metabolism is apparent in *P. stuartii*, where the chromosomal *aac(2')-Ia* encoding acetyltranferase is intrinsic in *P. stuartii*, which is transcriptionally regulated by number of genes (49-52). The mutations in any of the regulatory gene can lead to increased expression of *aac(2')-Ia* and increased aminoglycoside resistance. Clarke *et. al.,* discovered that O-acetylation of peptidoglycan is carried out by AAC(2')-Ia enzyme. Mutants of regulatory genes of this enzyme which overexpresses AAC(2')-Ia enzyme showed increased o-acetylation and evidently exhibited altered morphology

due to altered autolytic activity which depends on O-acetylation of peptidoglycan. The AAC(2')-Ia enzyme can acetylate aminoglycosides using either acetate from acetyl-CoA or peptidoglycan fragments or from N-acetyl glucosamine. Other potential role of AAC(2')-Ia is also lipopolysaccharide biosynthesis. Aminoglycoside modification by AAC(2')-Ia enzyme might be an unanticipated secondary effect rather than a primary effect (53, 54). In *E.coli*, and *P. stuartii*, overexpression of AarP (activator of 2-N-acetyltransferase [encoded by aac(2')-Ia]) resulted in multiple antibiotic resistance. AarP was found to be similar to MarA and SoxS proteins thus its overexpression also resulted in activation of the endonuclease IV gene (*nfo*), a gene in the SoxRS regulon of *E. coli* (55). Moreover, substrate specificity also plays an important role in determining resistance profile as seen in case of 6'-N-acetyltransferase family (AAC-6') which acetylates kanamycin, tobramycin, netilmicin, and sisomicin. AAC(6')-I subfamily of enzyme confer resistance to amikacin and C1a and C2 gentamycin while AAC(6')-II confers resistance to all isoforms of gentamycin but not to amikacin. Rather *et. al*., have showed that enzymatic activities of AAC(6') is affected by amino acid modifications at different positions. The decisive role of substrate specificity lies in presence of amino acid at 119^{th} position (leucine for amikacin resistance and serine for gentamycin resistance). Thus single point mutation is sufficient to change the resistance profile (56).

In the present chapter attempts were made to uncover, although unsuccessfully, the nature of apparently unusual apramycin resistant mutation that effectively explain one step high level resistance to apramycin and uncompromised growth of overproducer mutant. In the context of the known mechanisms of resistance discussed above, we present arguments which explain the probable mechanism of resistance to apramycin in #OP - (i) Apramycin modification by acetylation, phosphorylation, and adenylation which makes the antibiotic inactive may explain enzyme-mediated resistance to antibiotic, however unless the apramycin modifying function is also a transcriptional regulator, cannot account for pleiotropic phenotypes as in #OP; (ii) efflux function: the resistance pattern for #OP is specific to range of aminoglycoside antibiotics and does not conform with the nonspecific efflux mechanism for resistance to unrelated antibiotics; (iii)16S rRNA mutations or

modifications - single step high level resistance mutation in 16S rRNA is unlikely the reason given multiple copies of 16S rRNA genes in the genome of several free living eubacteria including *Streptomyces*. Among those *Actinobacteria* which produces aminoglycosides, posttranscriptional modification of the 16S or 23S rRNA are commonly observed including that in *Streptomyces* species and *Micromonospora* species. They are naturally resistant to the metabolites of their own. This process is mediated by posttranscriptional methylation by various 16S rRNA methyltransferases (16S-RMTases). If it is the case in #OP as well, in addition to resistance to different aminoglycosides, an alteration in the ribosome function may explain growth and chromomycin overproduction phenotype of #OP. Identification and cloning of the gene for resistance is expected to be possible in a heterologous host (37). Our inability to clone the candidate gene for methyl-transferase could be possibly due to the #OP library preparation being inadequate and not representative.

The function of chromosomally encoded aminoglycoside resistance provides an interesting case for consideration of the possibility of housekeeping genes' second function to be important in apramycin resistance. The annotated whole genome sequence of #OP showed presence of one aminoglycoside acetyltransferase and 3 putative aminoglycoside phosphotransferases genes. The possibility is open for transcriptional activation of any of the aminoglycoside modifying genes which may result in apramycin resistance and chromomycin overproduction. This mechanism could also explain the smooth colony morphology of #OP as it is observed in *P. stuartii* where overexpression of acetyltransferase resulted in altered cellular morphology. Substrate specificity of the modifying enzymes may only explain partial effects of the mutation but not all the pleiotropic phenotypes of #OP.

Mutation in elongation factor can also explain the phenotype of chromomycin overproduction and apramycin resistance in #OP possibly due to altered translatibility of certain mRNAs by the mutant ribosome and also for being *trans* recessive. Another likely mechanism is the mutation in the ribosomal protein gene which is also *trans* recessive to the wild type allele, and is supported by the effect of other ribosomal drug resistant mutations on antibiotic overproduction (14). Interestingly, generation of spontaneous resistant mutants for antibiotics acting on ribosomes such as

streptomycin and amikacin completely suppressed the resistance to apramycin and also overproduction of chromomycin (Fig 7.12; 7.13), on the other hand, erythromycin and paramomycin resistant mutants of #OP were not affected for either of the two phenotypes. Although, streptomycin and paramomycin bind to the same S12, the phenotypic suppression is limited to streptomycin alone. The proposal cannot stand the ground as the gene for S12; *rpsL* is not altered in the #OP. The apparent suppression of apramycin resistance by sreptomycin and amikacin resistance mutation each is also compatible with a mutationally altered substrate specificity of an aminoglycoside modifying enzyme.

In summary, the unique characteristics of #OP in relation to other mutations causing overproduction of antibiotics, since important for biotechnological applications include –

(i) ~176 fold oversynthesis of chromomycin in #OP which is comparable to the octuple mutant of *S. coelicolor* (~180 fold) containing a combination of 8 different mutations causing overproduction of the polyketide actinorhodin.

(ii) Growth rate of the mutant is not compromised, a feature that is commonly associated with the antibiotic resistant mutations.

(iii) Mechanism of genetic suppressions by streptomycin resistant mutation of chromomycin overproduction and apramycin resistance in #OP is unique not involving S-12 gene. Unlike in other cases, streptomycin and amikacin resistant mutation in the wild type each suppresses chromomycin synthesis. These and other characteristics indicate possible unique mechanism worth exploration.

(iv) Insertion mutant, *sflE*::tsr^{r} abolishing chromomycin biosynthesis disrupted for glucose 4,6-dehydratase function (*sflE*) of chromomycin biosynthetic cluster was same as #OP for all the phenotypes except of course chromomycin production, indicating that apramycin resistance mutation suppresses chromomycin requirement of WT for viability in stationary phase and physiology. A ‘cleaner’ marker free mutant deleted for the entire cluster of chromomycin biosynthesis genes in #OP is required to assess the potential of

this host for secondary metabolite production and is worth a comparison with mutants generated for this purpose using ribosome engineering strategy.

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