Part- A

Functional Analysis of Genes for Chromomycin Self-Resistance

INTRODUCTION

Antibiotic-producing micro-organisms protect themselves from the inhibitory/lethal action of antibiotics they produce by developing self-protection mechanisms (Martin et al., 2005; Mendez & Salas, 2001). Antibiotic self-resistance by the producer is achieved by various mechanisms which include drug extrusion, drug modification, target modification, and drug sequestration. (Biggins et al, 2003). These mechanisms need not be mutually exclusive and organisms make use of more than one such means for self defense. For e.g. *Streptomyces venezuelae*, producer of D-Desosamine, exports inactive prodrug which is acted upon by desR to make it active. But this forms only the secondary line of defense for the organism. Modification of 23S rRNA, which is the target site for methymycin and its derivatives, by PikR1 and PikR2 is a primary self-resistance mechanism. (Zhao et al, 1998)

Intrinsic ability of *S. flavisclerotícus* to withstand deleterious effects of its own product – chromomycin is attributed to the genes present in its chromomycin biosynthesis cluster. Cluster sequencing and subsequent analysis had revealed presence of three putative proteins (SfrA, SfrB and SfrX) probably involved in imparting self-resistance against chromomycin. Genes *sfr*A and *sfr*B encode for an ABC transporter system, which is a drug efflux mechanism, and *sfr*X encodes a UvrA-like protein of ABC excision nuclease systems, which are responsible for DNA repair (Chapter-6). Similar to regulatory proteins (SflRI and SflRII), these are the proteins that do not play direct role in biosynthesis of the polyketide molecule *per se*. While the former controls production of chromomycin, the latter ensures producer's survival in presence of chromomycin.

STRATEGIES & CONSTRUCTS

7a.2.1: Clone construction for resistance gene(s) expression in different combinations in heterologous host:

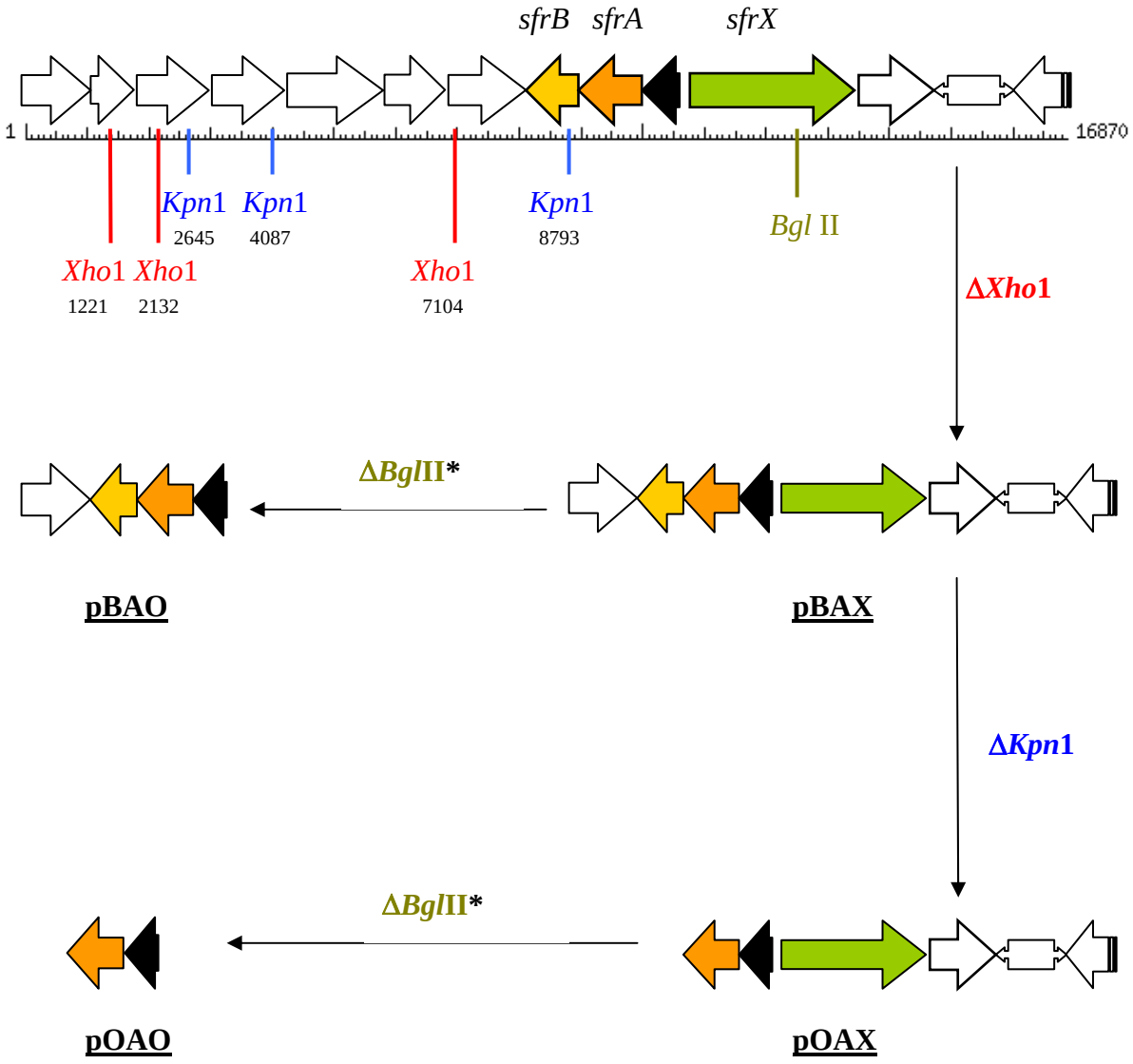

Fig7a.1: Schematic presentation of 17kb EcoR1 fragment, containing resistance genes, with restriction sites of *Kpn*1, *Xho*1 and BglII represented on nucleotide scale.

Restriction site mapping of p17EBSK using NEBCUTTER V2.0 software (http://tools.neb.com/NEBcutter2/index.php) showed presence of three *Xho*1 sites (1221, 2132, 7104) and three *Kpn*1 sites (2645, 4087, 8793) in the insert. All *Xho*1 sites are present upstream of resistance genes. Of the three *Kpn*1 sites, the last one (8793) is present within *sfr*B ORF and a *Bgl*II site is located at 14,188 internal to *sfr*X. To study functional attributes of resistance genes, p17EBSK was variedly pruned with different restriction enzymes to generate reduced clones with different combinations of relevant genes.

pBAX1 : Reduction of p17EBSK at *Xho*1 would delete 7.1kb excess DNA from insert to create pBAX. Fusion of the pBAX at *Eco*R1 site of pSET152 would yield pBAX1 wherein all the three genes involved in resistance remains intact.

pBAO1: Digestion of pBAX with *Bgl*II disrupts *sfr*X and fusion with *Bam*H1 site of pSET152 would maintain only *sfr*A and *sfr*B intact.

pOAX1: Reduction of pBAX at *Kpn*1 site would delete a portion of *sfr*B, to create pOAX. Fusion of pOAX with pSET152 at *Eco*R1 site would yield pOAX1. The clone contains intact *sfr*X and *sfr*A, but disrupts *sfr*B of two component system SfrAB.

pOAO1: Digestion of pOAX at *Bgl*II site and subsequent fusion with pSET152 at *Bam*H1 site would disrupt *sfr*X, maintaining *sfr*A as the only intact gene from the resistance cassette.

7a.2.2 Transformation:

All the four clones were transformed in *E. coli* S 17.1 and selection was done using ampicillin+apramycin markers. For p11ESET and control- pSET152 only apramycin selection was done. Since entire expression unit was in pBSK, it's orientation in pSET152 was not important. Confirmed tranformants was subsequently conjugated with *S. lividans* TK24 as described in Chapter-2.

RESULTS & DISCUSSION

There are two key advantages while working with genes involved in imparting resistance against a molecule. First, since they have a role of imparting resistance they do not depend on expression of other polyketide biosynthetic genes, and thus can be studied in isolation. This is not true with most other genes that form part of polyketide cluster. Secondly, they impart a phenotype when expressed. So, no markers are required for detecting expression levels of these genes nor does it involve any analytical technique to determine extent of effect attributed to the protein in study. These two features make them one of the best candidates for studying in heterologous systems. In particular, with earlier experience of poor transformation efficiency with *S. flavisclerotic*us, homologous recombination mediated disruption of resistance genes could have been a hurdle. Thus, heterologous expression of these genes in *S. lividans* TK24 – a well worked out strain routinely used for such studies was used to determine functional attributes of these putative proteins.

Divergent ORFs of *sfr*A-*sfr*B and *sfr*X provided an extra advantage in construction of clones. Since the common intergenic region with promoters was present between *sfr*A-*sfr*B and s*fr*X, pruning of p17EBSK for different constructs did not affect promoter region. Convergent or unidirectional position of genes would have eliminated promoter region at some step, probably demanding more efforts in clone constructions.

Preliminary results of earlier studies done in our lab, prior to sequencing of cluster DNA, had suspected presence of some resistance determinants in 11kbE fragment also (Namita Kumari, 2007). Sequencing results later, did not reveal presence of any complete ORF showing homology to PKS genes in 11kbE fragment. The possibility of its involvement in imparting resistance cannot be ruled out, particularly because this fragment contains NRPS genes and the cross-talk between PKS-NRPS is well documented (Beyer et al, 1999). Several naturally occurring molecules are hybrid products of NRPS and PKS are genes present in a cluster (Marahiel et al, 1997; Cane and Walsh, 1999; Du et al, 2000).

Fig-7a.2: Relative zone of inhibition in p11ESET (left) and control-pSET152 (right) when tested with crude extract (K20ul, K30ul) of *S. flaviscleroticus* and pure chromomycin (C10, C20)

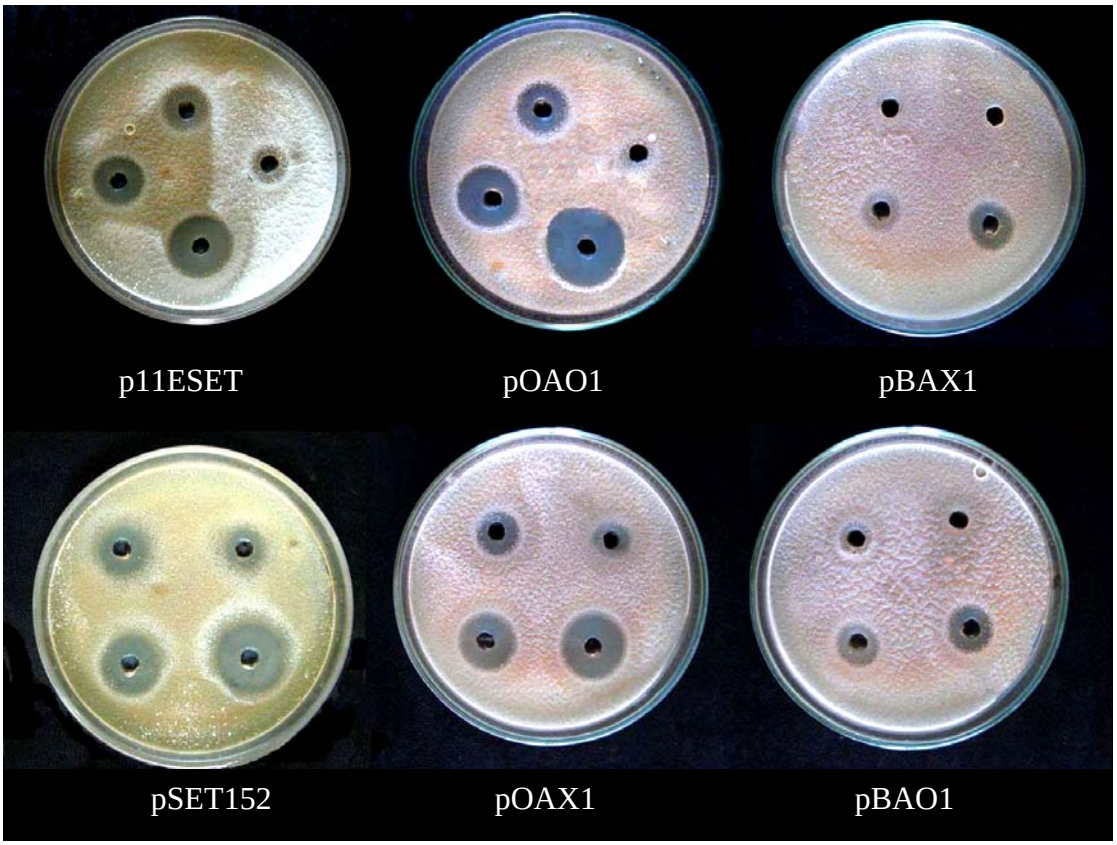

Fig-7a.3: Phenotypic characterization by expressing *sfr*B, *sfr*A and *sfr*X in hoterologous host

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Table-7a.1: Functional determination by zone of inhibition study against chromomycin, for various resistance determinants.

S. lividans	**Zone of inhibition (cm) at different chromomycin concentration**			
	5	**10**	**15**	**25**
pSET152	1.3	1.6	1.9	2.2
p11ESET	1.1	1.4	1.7	2.1
pBAX1	0	0	1.1	1.4
pBAO1	0	0	1.3	1.5
pOAX1	1.1	1.3	1.6	1.9
pOAO1	1.1	1.5	1.9	2.2

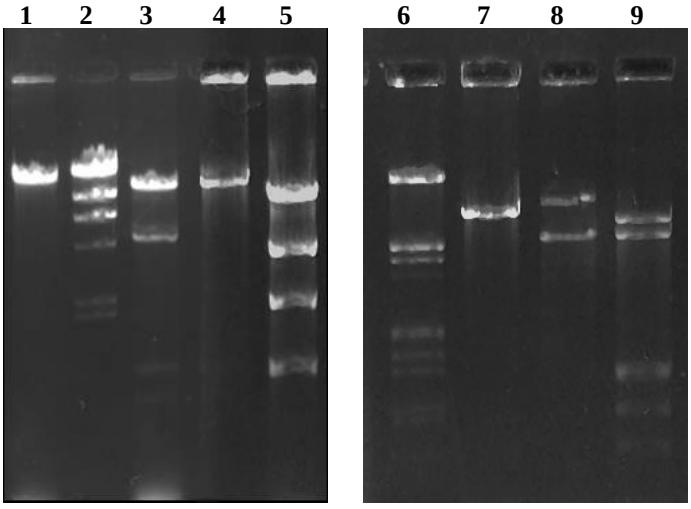

Fig-7a.4: Confirmation of various clones by checking restriction digestion pattern. (1) p17EBSK (2) $\lambda_{H\ marker}$ (3) p17EBSK digested with *Xho*1 (4) pBAX digested with *Xho*1 (5) p17EBSK digested with *Kpn*1 (6) λ_{HE} (7) pOAX digested with *Kpn*1 (8) pBAX1 digested with EcoR1 (9) pOAO1 digested with *Bam*H1.

Earlier doubt of 11kbE fragment bearing resistance determinants was ruled out in the present study. Transformants carrying p11ESET could not withstand chromomycin and further, there was practically no difference in inhibition zone when compared to control pSET152 (Fig- 7a.2). This result was not surprising particularly in light of the evidences obtained in support of horizontal acquisition of this PKS cluster discussed in earlier chapters. Since chromomycin cluster was a foreign DNA, recently incorporated in genome, one may not expect any resistance determinant to evolve elsewhere in the genome.

Further, the 11kb fragment was tested against the crude extract of *S. flavisclerotic*us to check if any of the genes in the fragment could confer resistance to other bioactive molecules produced by this organism. There was no difference in sensitivity of transformants with 11kbE and 17kbE fragments. Since chromomycin is produced in trace amounts, its contribution to crude, in comparison to other molecules, is very less. Thus, p17EBSK transformant gave inhibition zone equivalent to 11kbE. This also proves that the resistance genes of this acquired polyketide cluster cannot act against other bioactive molecules produced by *S. flavisclerotic*us.

Agar well assay carried out for all clone constructs revealed interesting results (Table- 7a.1). Clones (pBAX1 and pBAO1) where *sfr*A and *sfr*B remained intact showed a high tolerance to chromomycin. The resistance imparted was comparable to that of wildtype (Namita, 2007). On the other hand, SfrX showed a limited level of resistance as reflected by pOAX1 clone. Resistance shown by SfrX was no where comparable to that of SfrAB and appeared to be weaker mechanism of resistance, in isolation (Fig- 7a.3). Based on these observations, it can be easily concluded that SfrAB forms primary mechanism of chromomycin defense in *S. flavisclerotic*us, whereas SfrX appears to be playing a supportive role or is probably used as a backup system by the organism.

Similar work has been published recently (Menendez et al, 2007) by a group working on other chromomycin producer, *S. griseus* sub *griseus*. We differ in our approach towards proposed functioning of resistance genes. Both the mechanisms, suggested by them are preventive in nature. We believe that while the transporter has a preventive mechanism

of action, SfrX plays a curative role. It has been shown that *S. griseus* secretes a relatively inactive compound, DDACA3 (4A,4E-O-DiDeacetyl-Chromomycin A3) and its activation on cell surface (CmmA) is followed by its export via efficient machinery (CmrAB). This provides an advantage to the producer strain, to survive during antibiotic biosynthesis. But in that case chromomycin sensitivity of SfrAB is obscure. Though the cell exports prodrug, the active drug is always present in its environment. As in any other cell, it will passively enter back into producer cell as well. The event is expected to occur rather, relatively at higher rate due to the concentration gradient which will be highest in producer's vicinity. At this point, due to sensitivity of SfrAB towards chromomycin, it will be effluxed out of the cell, thereby imparting self resistance. SfrAB plays a preventive role by keeping chromomycin out of reach of its target i.e. chromosome (DNA). The higher sensitivity of SfrAB (as has been suggested for CmrAB) towards DDACA3 compared to chromomycin is also justified owing to very high intracellular concentration of DDACA3 produced by cell against chromomycin, which enters the cell passively via diffusion. Thus, it is necessary that SfrAB should be looked upon simply as an exporter of some molecule (DDACA3) and be distinguished from its ability to impart resistance against chromomycin.

In light of the proven ability of this highly efficient pump to extrude a relatively inactive prodrug out of the cell, proposed mechanism for CmrX action by Mendez et al, is not very convincing and is practically unacceptable. They hypothesize CmrX to play a preventive role by competitively binding to the chromomycin target site (DNA). Such mode of action would require synthesis of abundant CmrX to ensure effective coverage of minor grooves (site of chromomycin action) throughout genome. This is an enormous load to the cell in terms of energy and aminoacid pool utilization particularly when firstly, the protein is relatively bulky (826 amino acids) and secondly, when the molecule in intracellular environment is not in its matured active form. We hypothesize that SfrX has a curative role to play in imparting self resistance to the cell. The passively entering chromomycin molecules that escape SfrAB action would bind to the DNA and damage it. SfrX which has high homology to Uvr –A like DNA damage repair proteins, would act on such minor damages and repair them. This theory is supported by proposed self resistance mechanism of analogous MtrX (Garcia-Bernardo et al, 2000), to which SflX shares highest homology.

Also, low level of resistance imparted by SfrX, when expressed alone (pOAX1), can be convincingly explained by this hypothesis, which is not possible to do if a preventive role is assigned to SfrX.