Part- B

Functional Analysis of Key Regulatory Gene (sflRI) & its Promoter (actRp)

INTRODUCTION

The biosynthesis of secondary metabolites under laboratory conditions usually occurs late in a growth phase or developmentally controlled manner. It is also influenced by a wide variety of environmental and physiological signals, presumably reflecting the range of conditions that trigger their production in nature. The expression of secondary metabolite gene clusters is controlled by many different families of regulatory proteins, some of which are found only in *Actinomycetes*, and is elicited by both extracellular and intracellular signaling molecules. Many of the pathway-specific regulatory proteins that control secondary metabolism in *Streptomycetes* belong to the SARP (Streptomyces Antibiotic Regulatory Protein) family. They have been found associated with secondary metabolite gene clusters that encode aromatic polyketides (Sheldon et al, 2002; Ichinose et al 2003; Pang et al, 2004), ribosomally and non-ribosomally synthesized peptides (Ryding, 2003), undecylprodiginines (Cerdeno et al, 2001), Type I polyketides [Sun et al, 2003; Oliynyk et al, 2003), β-lactams (Nunez et al, 2003) and azoxy compounds [Garg et al, 2002]. While genes encoding phylogenetically diverse classes of bacterial regulatory proteins occur in many secondary metabolite gene clusters, the SARP family of proteins has only been found in *Actinomycetes*, and most of them within the *Streptomycetes*

In silico analysis of the sequencing results of chromomycin cluster suggested, presence of two putative regulatory genes (Chapter-6). Of the two, SflRII resembles to a negative regulator belonging to *pad*R family proteins (Gury et al, 2004) and has been recently studied in *S. griseus* sub *griseus* chromomycin cluster. SflRI, another protein in the culster, in our analysis showed a conserved domain of SARP family of proteins. These SARP family proteins play key role in recruiting RNA polymerase to recognize weak promoters of PKS cluster and thereby facilitate transcription initiation of the cluster, in dose dependent manner (Tanaka, 2007).

A complex region containing SflRI promoter (*actRp*) was also determined bioinformatically. It was cloned with EGFP (Enhanced Green Fluorescence Protein) marker

to obtain further insights about the conditions that influences its induction. Optimization of these conditions may increase SflRI synthesis thereby influencing chromomycin production. SflRI in its role as a regulatory protein appears to perform a key task in chormomycin production, by directly not getting involved anywhere in biosynthesis, but rather by governing entire process of molecule biosynthesis. SflRI expression might also have a bearing in counteracting suppression by SflRII. Silencing of a gene involved in biosynthesis is expected to affect the architecture of the molecule and not production. While disruption of *sfl*RI would not cause any alteration in chromomycin structure but would entirely abolish production of the polyketide.

STRATEGIES AND CONSTRUCTS

7b.2.1 Construction of *actRp*-EFGP transcriptional fusion:

A 0.7kb *Bam*H1-*Eco*R1 fragment containing *actRp* region was released from the p2.2BBSK clone, by ustilizing *BamH*I site in the MCS of the backbone and an *Eco*RI site present upstream of activator gene. The promoter to be cloned in pIJ8660 was first cloned in pBSK and after transformation, purification and subsequent plasmid isolation insert was reconfirmed by PCR using T3 and T7 primers. The recombinant clone was digested with *Bam*H1 and *Kpn*1. Presence of these sites on opposite sides of *Eco*R1 in MCS of pBSK and absence of any *Kpn*1 site in the insert, ensured release of complete insert. *Bam*H1-*Kpn*1 fragment was cloned in the corresponding sites present in the MCS, upstream of EGFP gene of pIJ8660 (Fig-7b.1A). For clone confirmation, the construct was digested with *EcoR*1 to release 1.8 kb fragment generated by a site in backbone and a site in the insert (Fig- 7b.1 B).

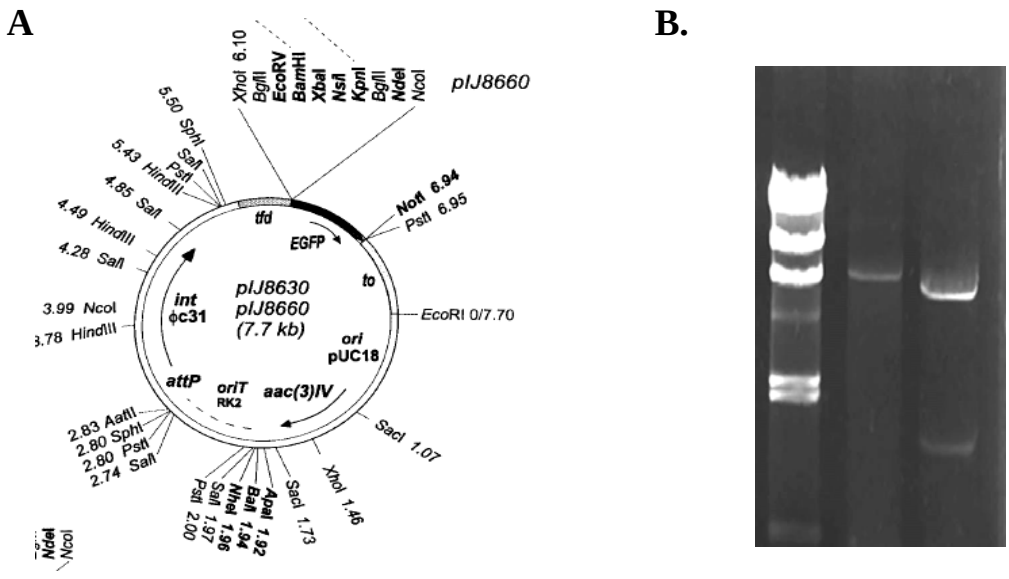

Fig-7b.1 : Clone construction and confirmation of *actRp*-EGFP transcriptional fusion system. A) Map of promoterless EGFP containing pIJ8660; B) 1. λ_H marker 2. *actRp*pIJ8660 linearized with *Bam*H1 3. 1.8kb *Eco*R1 insert release from the same clone.

7b.2.2 Introduction of Clone:

The ligation mixture was transformed in *E. coli* S17.1 strain, for conjugation, and selection was done using apramycin marker. Replica plating on ampicillin plate was done to eliminate false positives generated by ligation of pBSK and pIJ8660. Clone confirmation was done by *Eco*R1 digestion of isolated plasmid from purified colonies. The non-recombinant clones would get linearized while the recombinants would release a fragment of 1.8 kb, due to the presence of *Eco*R1 site in the insert.

Intergenic conjugation between *E. coli* S17.1 and *S. lividans* TK24 could be achieved at a very high frequency by following the prescribed protocol for spores as well as for mycelium. For *S. flavisclerotícus,* use of mycelium was avoided since it yielded poor results over spores. Albeit with spores, number of exconjugants obtained per plate was less than that of TK24, the results were convincingly reproducible.

Similarly, transformations of pIJ8660 and pIJ8655 in *E. coli* S17.1 and subsequent conjugations in TK24 and *S. flavisclerotícus* were also carried out. Promoterless pIJ8660 was used as a negative control in the experiment whereas pIJ8655 with a *tipAp* cloned upstream of EGFP was used as a positive control.

7b.2.3 Simultaneous gene disruption and complementation strategy:

A modification in conventional gene disruption strategy was made, to carry out disruption as well as gene complementation in a single crossover event. For this, a truncated gene (*sfl*RI) with its intact 5' terminal was amplified. This fragment was appropriately cloned downstream of an inducible promoter (*tipAp*) in suitable vector (pIJ8600ΔH).

On transformation in the host (*S. flavisclerotícus*), in absence of site specific machinery (*att -int*), the suicidal plasmid (pSET152) is expected to undergo recombination in the host genome using insert (*sfl*R1) homology as shown in (Fig- 7b.2A). This will lead to

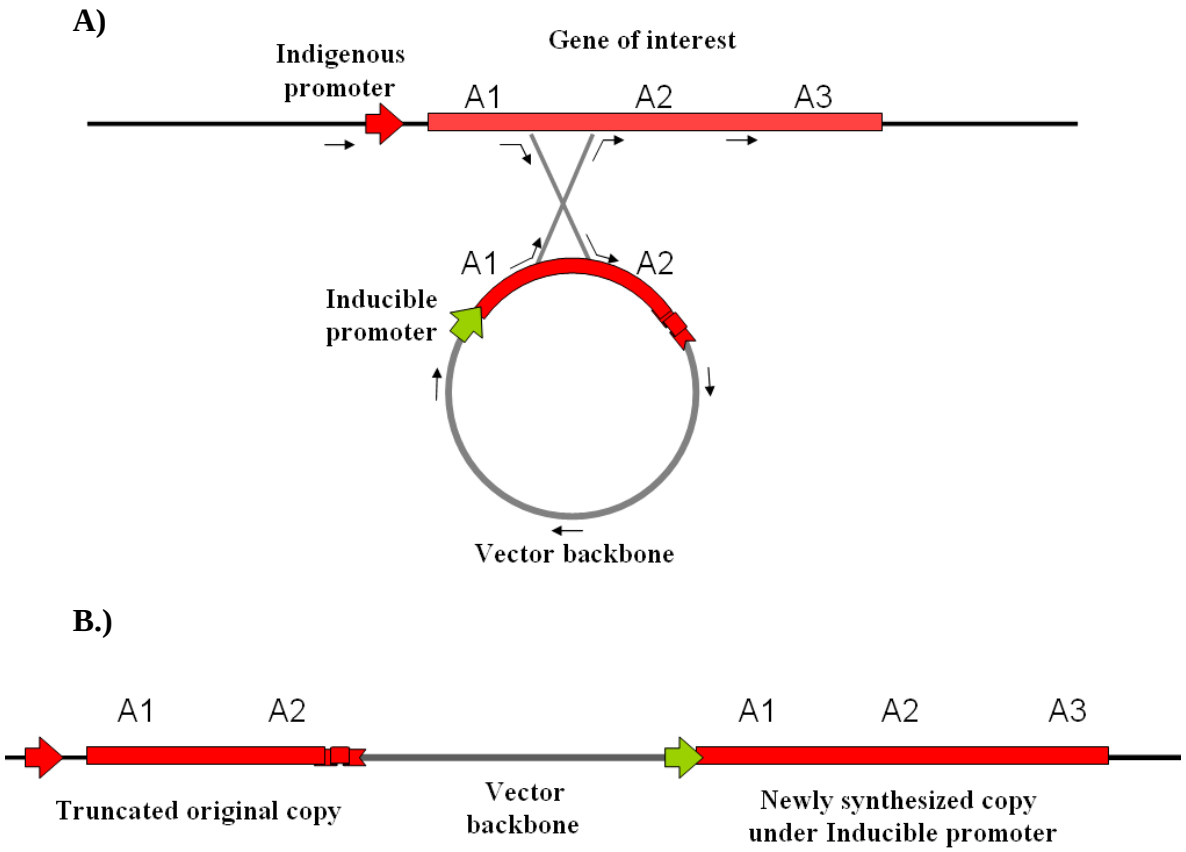

Fig-7b.2: Diagrammatic representation of the proposed strategy. A) Cross over event between plasmid insert under inducible promoter with genomic copy. B) Post-recombination changeover in the genome.

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disruption of genomic copy of gene under its original promoter and a new functional copy of gene will be formed downstream of vector, which shall be under control of inducible promoter (*tipAp*) as shown in (Fig- 7b.2B).

Modification of pIJ8600 for homologous recombination:

Site specific recombinations are highly efficient as compared to homologous recombinations and thus would mask latter event in the cell. Thus the *att* site and *int* gene present in pIJ8600, responsible for site specific recombination, were removed. This was

achieved by digestion of pIJ8600 with *Hind* III and subsequent re-ligation, to generate pIJ8600ΔH. In the process, the plasmid was reduced to 5.65 kb from its original size of 8.1kb, loosing 2.4kb. The 2.4kb DNA consisted of three fragments of 0.71kb, 0.8kb and 0.94kb containing a part of *int* gene region. Thus a truncated integrase gene would not contribute to the site specific recombination and the suicidal vector can now only get integrated in genome by homologous recombination. *Pst*I or *Sal*I could have been better candidates for the purpose, because they not only remove *att – in*t completely but also reduce the plasmid to ~4kb size. But the reduced plasmid would also lack *oriT* required for intergenic conjugation, eliminating option of conjugation, leaving only the option of transformation.

7b.2.2.2 Clone construction and verification:

An *sfl*R1 amplicon of 0.9kb was generated using specific primers (Fwd = Fact; Rev = Ract) designed using PRIMER-3 sofrware. The amplicon was digested with *Hind*III at 5' end and *Xho*1 internal site, present towards 3' end of the gene, and cloned in pBSK to form HXpBSK which was then fused with pIJ8600ΔH at *Hind*III site to form pTRACT (for truncated activator). Since complete MCS of pIJ8600 was removed, in formation of pIJ8600ΔH, the insert was cloned in pBSK and then fused with pIJ8600ΔH at *Hind*III site, as described in previous experiment. *E. coli* S 17.1 transformants were selected on ampicillin – apramycin double selection background. pTRACT ligation being non-directional, fusions could have occurred in either orientations generating two different clones (Fig – 7b.3). Utilizing *EcoR*1 internal site in the amplicon, clone with correct orientation was determined. Clone in correct orientation would establish truncated *sfl*R1 downstream to *tipAp*, releasing fragment of ~1.4kb when digested with *Eco*R1.

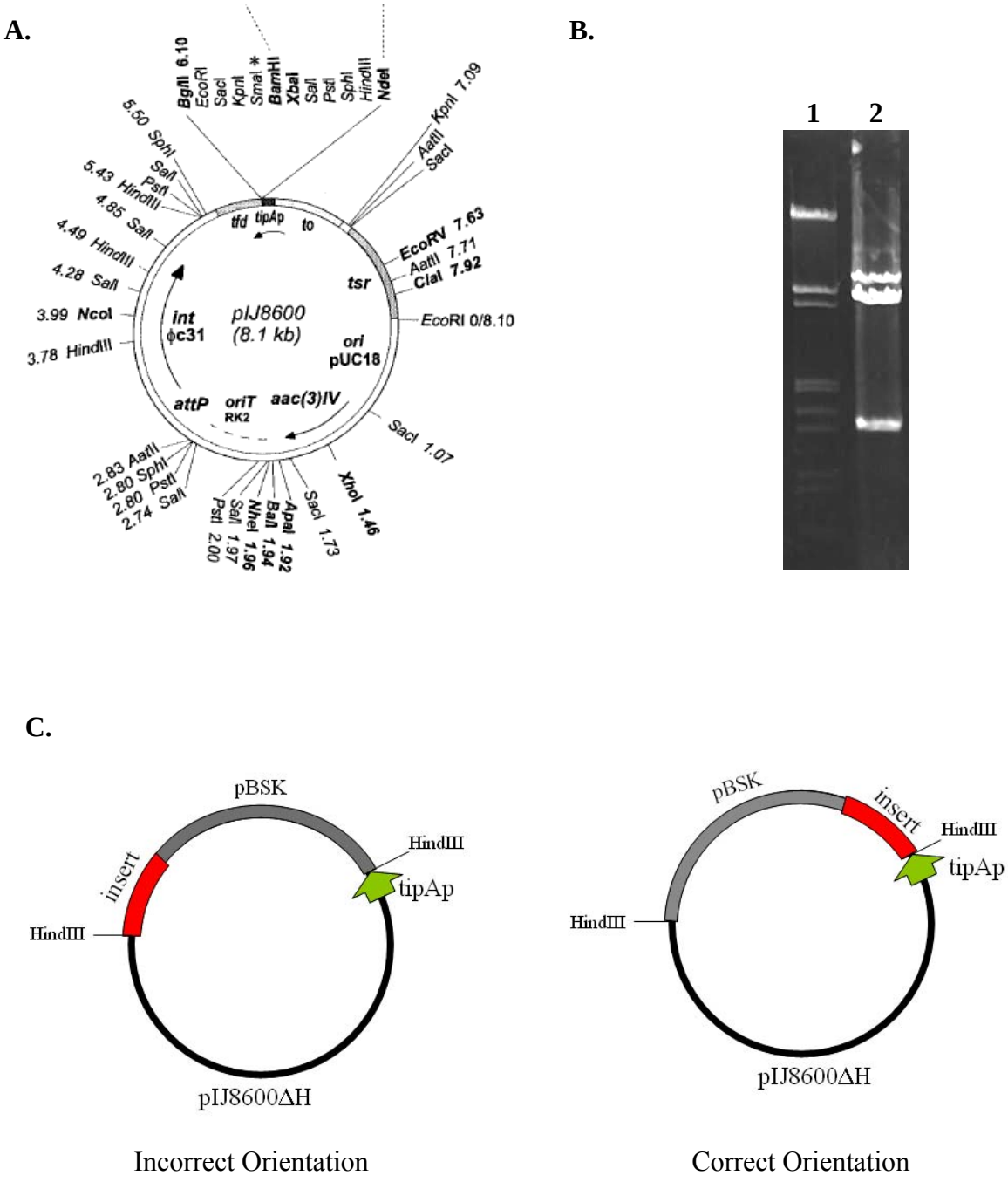


Fig-7b.3 : Construction and confirmation of pTRACT clone: A.) Pij8600 map B.) clone confirmation -1.λ_{HE} 2. *Eco*R1 digestion 3. pTRACT 4. *Sal*1 digestion. C.) Possible orientations of HXpBSK ligation with pIJ8600ΔH at *Hind*III.

RESULTS AND DISCUSSION

7.3.1 *In silico actRp* analysis:

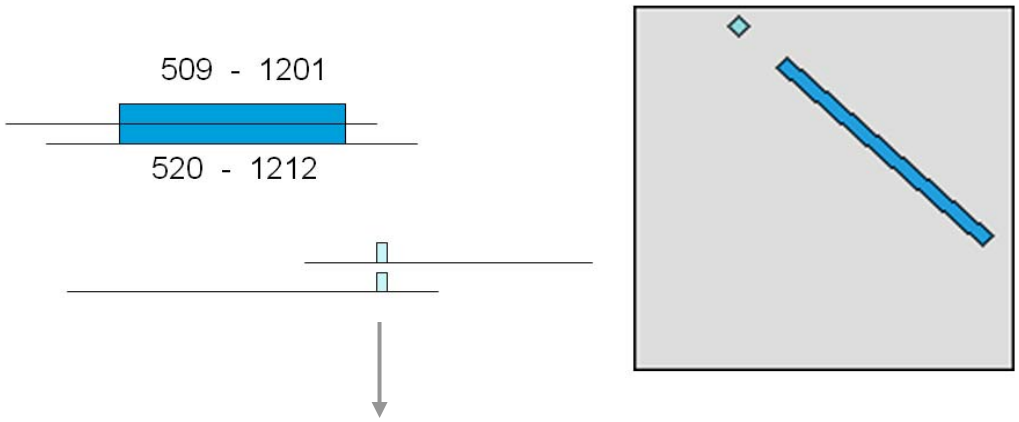
Based on the analysis of chromomycin cluster in Chapter -3, *sfl*RI is present between two methyl transferase genes, namely *sfl*MII and *sfl*MIII. Both these genes are placed divergent to this regulatory gene. Thus, it is for certain that *sfl*RI is not an intermediate gene of any operon, rather an independently transcribing gene with its own promoter.

Intergenic DNA of 526 bases present between start codons of *sfl*RI and *sfl*MII was analyzed for putative promoter region using BPROM software (www.softberry.com) and Neutral Network Promoter Prediction Software (http://www.fruitfly.org/seqtools/promoter.html) popularly used to fish-out prokaryotic promoters. Putative promoter regions were highlighted in both directions, though at a score of 6.3. Presence of promoter on minus strand could be due to presence of *sfl*MII in opposite direction to *sfl*RI. The relatively average score should not be considered as measure of promoter strength, particularly in this case, since the genus has GC rich genome and so is expected to show variation in its promoters. (Strohl, 1992)

The putative sequence for *actRp* is as follows:

GCCCGC**TTGACT**CATGAAACTAATCTCA**TGAAACT**AGTCG**T**GCGACTCTA

For further analysis, this upstream region along with activator gene was subjected to BLAST2SEQ analysis with its counterpart from *S. griseus*. As expected, both genes being analogous, showed very high homology score. But, surprisingly there was no homology between the intergenic DNA upstream of activator, except for a small patch of 34 bases (Fig- 7b.4). Sequence of the conserved island was found to be the same as that ascribed to be putative promoter for the activator gene.



S. flavisclerotícus : 339 CGCTTGACTCATGA**A**A**C**TAATCTCA**T**GA**A**ACTAG 372

S. griseus : 1378 CGCTTGACTCATGA**G**A**A**TAATCTCA**G**GA**G**ACTAG 1345

Fig -7b.4: Conserved island of promoter region in a totally non-conserved intergenic DNA upstream of activator gene in *S. flavisclerotícus* and *S. griseus*

It is obvious that non-coding intergenic DNA would accumulate all mutations without any bias but a functional ORF provides only a limited plasticity. So, under any given circumstances the coding DNA will always have less mutation accumulation than the intergenic DNA. A drastic difference in nucleotide conservation between the neighbouring regions (intergenic and activator) of the two species is clearly indicative of a very high mutational rate prevailing in these two species. We hypothesize that this could be due to a relatively higher mutational rate which might be prevailing in these species. The prediction is based on the fact that the cell continuously produces chromomycin and any lapse in resistance machinery would form a potential opportunity for mutation incorporation.

Thus presence of a small island of conserved region within totally non-conserved intergenic DNA advocates for its essential nature and asserts for its claim as promoter region.

Important features of *actRp*:

- Promoter region is identified 198 bases upstream of AUG translation site.
- As seen in table- 7b.1 TTGACT is identified as -35 box which matches with the concensus sequences TTGACA (Bourn and Babb, 1995).
- TGAAACT represents -10 box sequence (table- 7b.1), against the expected concensus sequence TAGR(G/T)T (R=A, C or G). (Bourn and Babb, 1995)
- While -35 box appears to be much conserved with σ^{70} of *E. coli*, -10box considered to be more important for σ factor recognization, is very different from that of σ^{70}.
- G+C content of the region was found to be 67% which is much high in comparison to *E. coli* promoters with 43% G+C content (Bibb et al, 1986)
- Unlike several *Streptomyces* promoters with a spacer region of 17 or 18 bases (Strohl, 1992), *actRp* followed convention of 16 nucleotides.
- An identical direct repeat of 12 bases involving -10box is present towards -35box, covering entire spacer region and includes 2 bases of -35 box.
- These direct repeat sequences contain a hexameric palindrome TCATGA within it
- There are two overlapping siamese inverted repeats from -3 to -22 and from -10 to -29.
- There is presence of another putative promoter on the opposite strand for methyl transferase (s*flMII*) gene.

-35 **-10** **+1** **promoter**

gcccgc**ttgact**catgaaactaatctca**tgaaact**agtcg**T**gcgactctattgg

ctcatgaaacta **ctcatgaaacta** **direct repeats**

actaatctca/tgaaactagt **inverted repeat**

tcatgaaact/aatctcatga **inverted repeat**

Table-7b.1: Relevance of *actRp* with consensus sequence (Bourn and Babb, 1995) and also with its counterpart from *S. griseus.*

	-37	-36	-35	-34	-33	-32	-31	-30	-29	-28	//
SF	G	C	T	T	G	A	C	T	C	A	
G	**7**	5			**15**			6	5	6	
C	5	**8**					**15**	3	**10**	5	
A	0	1				**15**		4		**1**	
T	3	1	**15**	**15**				**2**		3	
SG	G	C	T	T	G	A	C	T	C	A	

	-19	-18	-17	-16	-15	-14	-13	-12	-11	-10	-9	-8	-7
SF	A	T	C	T	C	A	T	G	A	A	A	C	T
G	16	18	19	18	29	16	**0**	**0**	24	**20**	25		21
C	23	13	**22**	15	**10**	17			18	17	8	**0**	18
A	**7**	7	3	3	6	**11**		52	**6**	**12**	**8**		3
T	6	**14**	8	**16**	7	8	**52**		4	3	11	52	**10**
SG	A	T	C	T	C	A	G	G	A	G	A	C	T

***In vivo actRp* analysis:**

During clone construction an extra step involving cloning in pBSK and subsequent release of the fragment was necessary to satisfy limited MCS of pIJ8660 as well as to ensure correct orientation of promoter upstream of EGFP gene. Intergenic conjugations using same volume of culture inocula resulted in an average 300 exconjugants of *S. lividans* TK24 on every plate against only 12 per plate with *S. flavisclerotic*us. Thus, *S. flavisclerotic*us showed a really very low exconjugant frequency even with site specific recombination.

The microscopic determination of both TK24 and *S. flavisclerotic*us containing different plasmids was done under phase contrast as well as fluorescence lamp. 40X magnification imparted adequate enlargement of mycelium, and was maintained throughout the study.

Fluorescence of cultures with pIJ8655, grown in increasing concentrations of thiostrepton (inducer), was measured by fluorescence analyzing software attached to microscope. Though *tipAp* has been reported to show leaky expression (Vierling et al, 2001), our result is in line with other results (Sun et al, 1999) where there was no detectable leaky expression of *tipAp* observed. *tipAp*, besides its sensitivity to thiostrepton, has also been shown to get induced with increase in osmolarity (Ali et al, 2002). The variable results of *tipAp* could be probably due to differences in osmolarity which the cell might be experiencing at that time.

A thiostrepton dose dependent increase in fluorescence emission was observed in the cultures which could be captured in the fluorescence microscopy and after software based intensity analysis, a linear standard graph could be plotted based on the data.

As a negative control, a pIJ8660 (with promoterless EGFP) containing exconjugant was also checked for fluorescence. Absence of any fluorescence ruled out scope of any background emissions or noise.

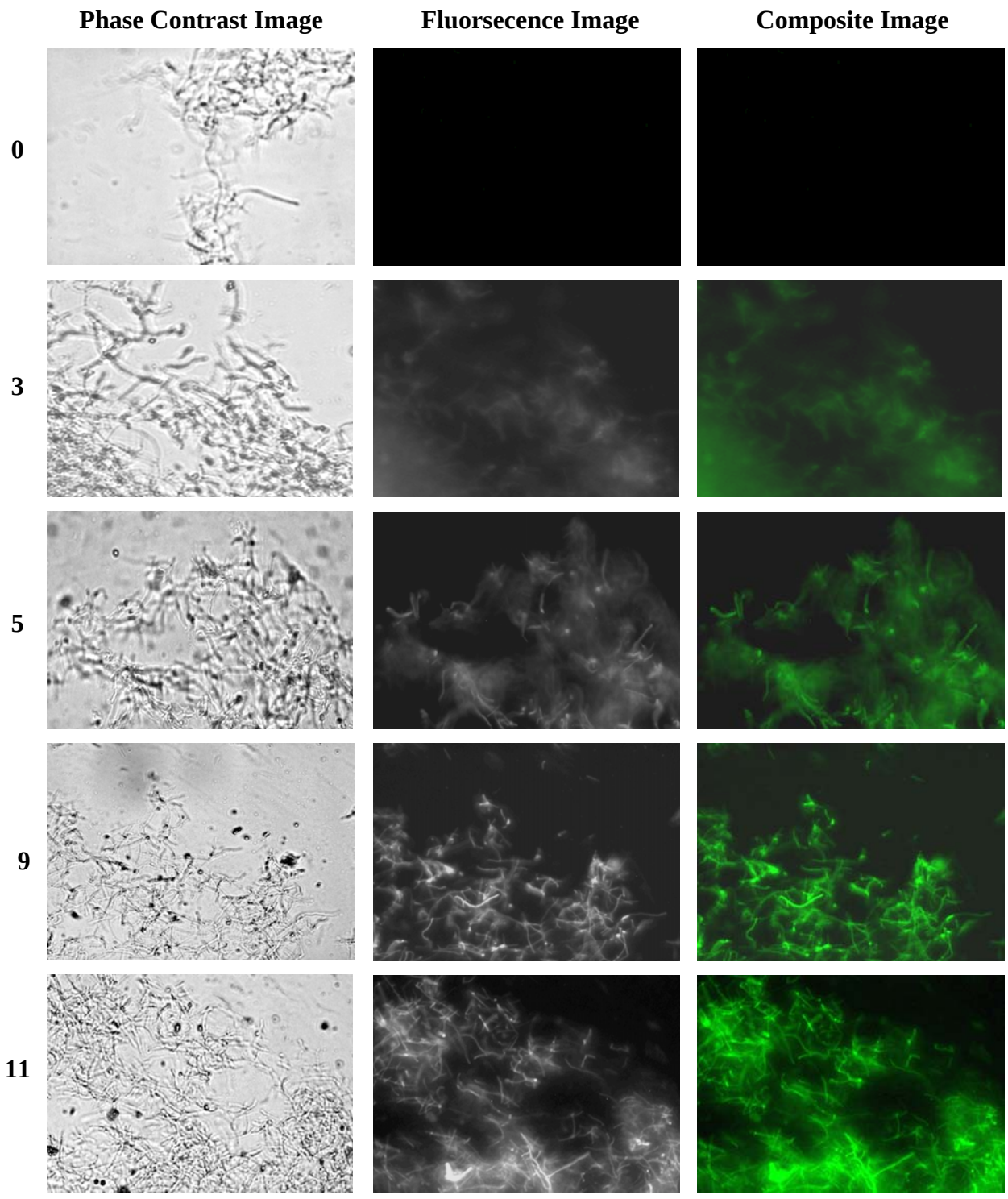


Fig-7b.5: Thiostrepton mediated *tipAp* induction in *S. lividans* transformed with pIJ8655 resulting in concentration dependend increase in fluorescence of EGFP.

The *actRp*-pIJ8660 clone, when transformed in *S. lividans* TK24 resulted in restricted fluorescence emission. It was observed that only the older mycelia fluoresced. This is justifiable, *actRp* being promoter for activator of PKS cluster which gets activated with onset of stationary phase, presumably via this SARP protein. The estimated fluorescence intensity measured, for 2 and 4 day old mycelia (Fig- 7b.6), was 56.6 units as analyzed by Image-ProPlus-5.1 softwar. This was equivalent to the fluorescence emitted by mycelium induced with ~ 5ug/ml of thiostrepton, (Graph- 7b.1).

Activity of *actRp* in heterologous host cannot be extrapolated for its actual performance in *S. flavisclerotic*us. To study the promoter strength in its native environment, the same clone (*actRp*-pIJ8660) was introduced in *S. flavisclerotic*us by conjugation, as described earlier. Interestingly no fluorescence could be detected for 10 exconjugants tested. In *S. lividans* fluorescence could be recorded on second day of inoculation and stabilized on fourth day when culture reached stationary phase. But in *S. flavisclerotic*us there was no change in results even after six days of growth. Results remained unaltered, even on using various media (LB, TSB, YEME, DNB, SM) and altering conditions like temperature (25 ^{o}C, 30 ^{o}C, 37^{o}C) and pH (6, 7, 8).

The possibility of fluorescence quenching at any stage in the cell environment was ruled out since results of pIJ8655 exconjugants of *S. flavisclerotic*us reproduced results obtained in *S. lividans*. Any doubt in clone construction was disqualified, since the same clone had already given positive results in *S. lividans*. The exconjugant was comparable to the *S. flaviscerloticus* wild type in all aspects of phenotype.

Disruption of *cmm*RII in *S. griseus* had led to an early onset of chromomycin biosynthesis and there was 70% increase in production as well. This confirmed role of cmmRII as a repressor of transcriptional regulator (Menendez et al, 2007). Lack of fluorescence with *actRp* in *S. flavisclerotic*us and presence of direct repeats in *actRp*, suggests that SflRII (analog of CmmRII) must be binding to these direct repeats of *actRp* to check transcription of SflRI. Ability of *actRp* to express in a heterologous host but not in native environment can probably be justified with this theory. Lack of any negative

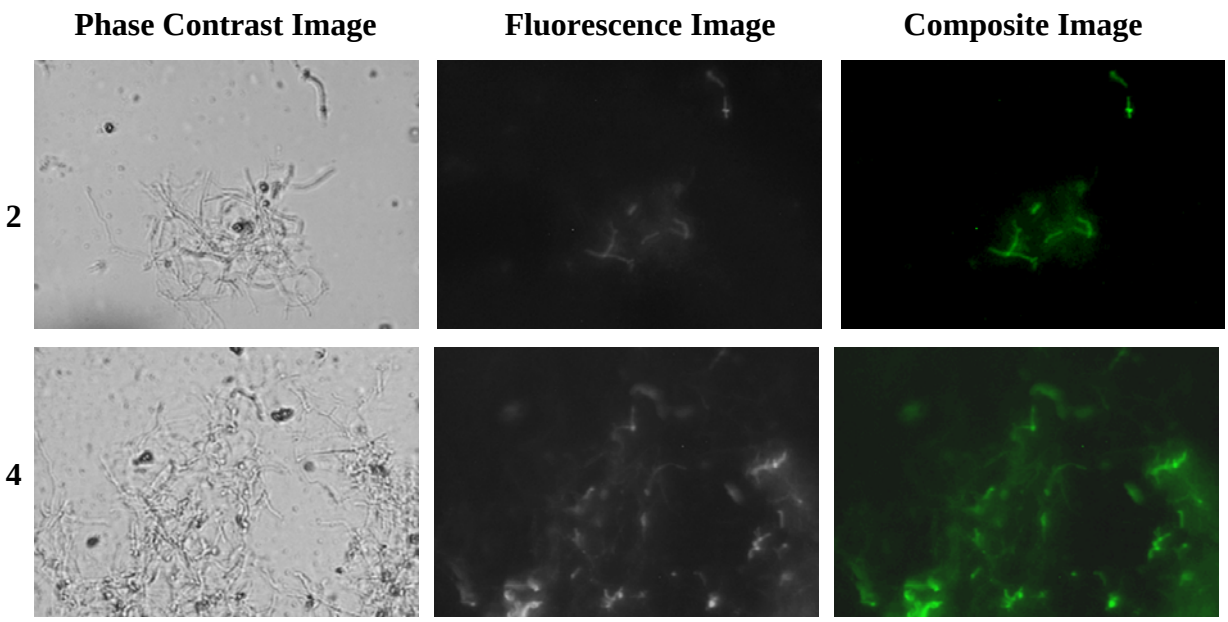
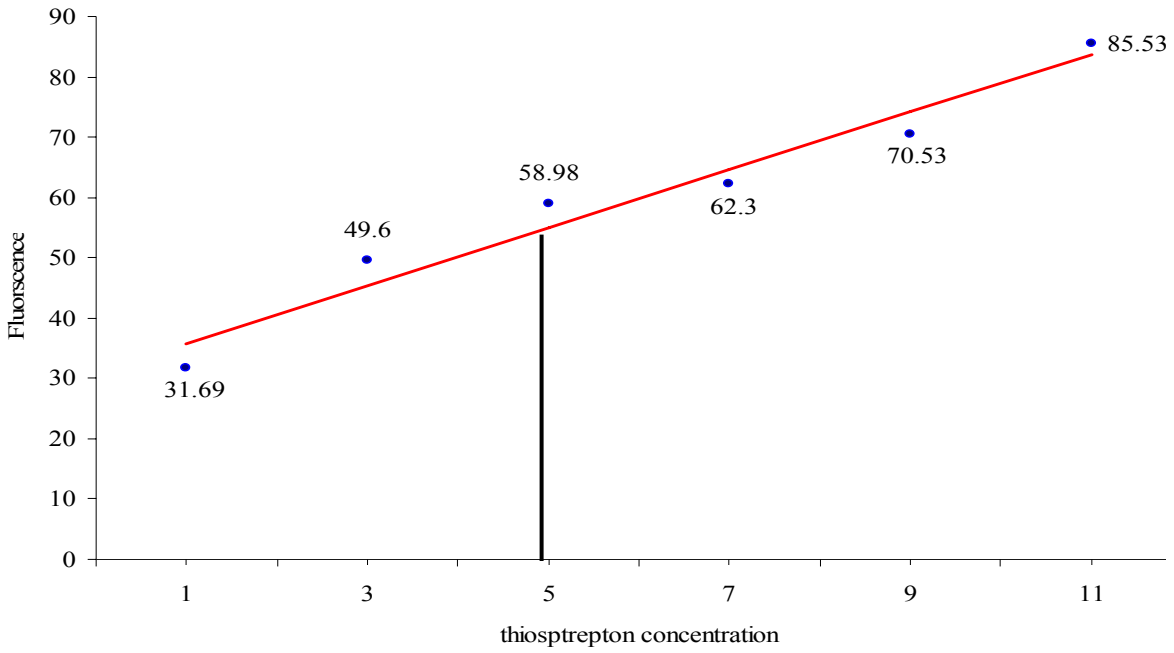


Fig-7b.6: Fluorescence detection in pTRACT transformed *S. lividans* after 2 days and 4 days of incubation. An overall increase in fluorescent mycelia was observed with time but the intensity remained constant.



Graph-7b.1: Relative promoter strength measurement of *actRp* by plotting fluorescence emission values of EGFP on standard graph generated using thiostrepton inducible promoter (*tipAp*) at increasing concentrations of thiostrepton.

regulator (SflRII) in heterologous host could have allowed the promoter to express and exhibit fluorescence and the presence of same in *S. flavisclerotic*us could have restrained it from expressing.

Unexpectedly in absence of fluorescence, secondary metabolite production in the exconjugant remained unaffected. This indicates that the original genomic copy of the promoter was functional and could transcribe *sfl*RI, finally resulting into chromomycin production, but only the introduced copy of *actRp* was not functional. In our literature survey we could not find any report wherein two copies of same promoter in the same cell responded differently. As stated earlier, the regulatory proteins besides regulating chromomycin biosynthesis, also appears to have a role is checking their own regulation. Interplay between SflRI and SflRII might be developing a dynamic equilibrium for controlled biosynthesis of chromomycin.

Based on the information so far generated, we hypothesized that interplay between SflRI and SflRII develops a dynamic equilibrium leading to a tightly controlled biosynthesis of chromomycin. For this, functional attributes needs to be assigned to SflRI. A dose dependent expression study for the protein would have probably revealed its role beyond activation of chromomycin biosynthesis.

_sfl_RI disruption in _S. flavisclerotic_us by homologous recombination

A revision in the method of clone construction could enable disruption as well as complementation of SflRI in a single recombination event. This eliminates entire effort incurred in SflRI complementation experiment, reducing the efforts to half. Necessary and sufficient conditions required for functional analysis of any gene could be governed by switching on/off the inducible promoter. Going a step further, the strategy would provide an exclusive advantage in studying functional role of an essential gene. In conventional strategy, disruption of essential gene would cause lethality and thus no transformants can be achieved (Parish and Stoker, 2000; Sassetti et al, 2001). But using this strategy, selection of transformants on media supplemented with inducer would enable the transformants to grow.

Inability of transformant to grow, later on media without inducer supplementation would confirm the clone/essential nature of gene.

Conventional strategy (Hopwood et al, 2000) requires amplification of an internal fragment, to be cloned in a suicidal vector. The clone is expected to undergo homologus recombination using the insert homology. But, this only brings about disruption of gene, thereby making it non-functional. For functional analysis, re-introduction (gene complementation) of functional gene also needs to be achieved, in order to replenish lost protein. Thus for this, complete gene needs to be amplified and cloned, in another vector with an ability to undergo site specific recombination and with different antibiotic marker. Re-transformation of the disruption mutant with this construct will restore activity of gene product. Thus, whole event involves two amplifications, two cloning and two transformations.

While in the modified strategy, disruption of native genomic copy under it's own promoter and formation of a new copy under an inducible promoter, is achieved in a single step of homologous recombination (Fig- 7b.2). The functionality of gene product is achieved by switching on/off the inducible promoter. Thus, the modification not makes the event easier and simpler but is also saves on time and efforts.

Further bioinformatic exploration of sequence data revealed presence of similar promoter complex between *sfr*X and *sfl*RII which was again much conserved with its counterpart from *S. griseus*.

SARPs are known to recognize and bind to such tandem repeats and thereby regulating the biosynthesis process. But in this case, both the regulatory proteins themselves show presence of such tandem repeats in their promoter regions. This suggests that the two regulatory proteins not only regulate chromomycin biosynthetic genes but also regulate each other. Thus, either protein might be binding to the tandem repeats and thereby control transcription of the other protein.

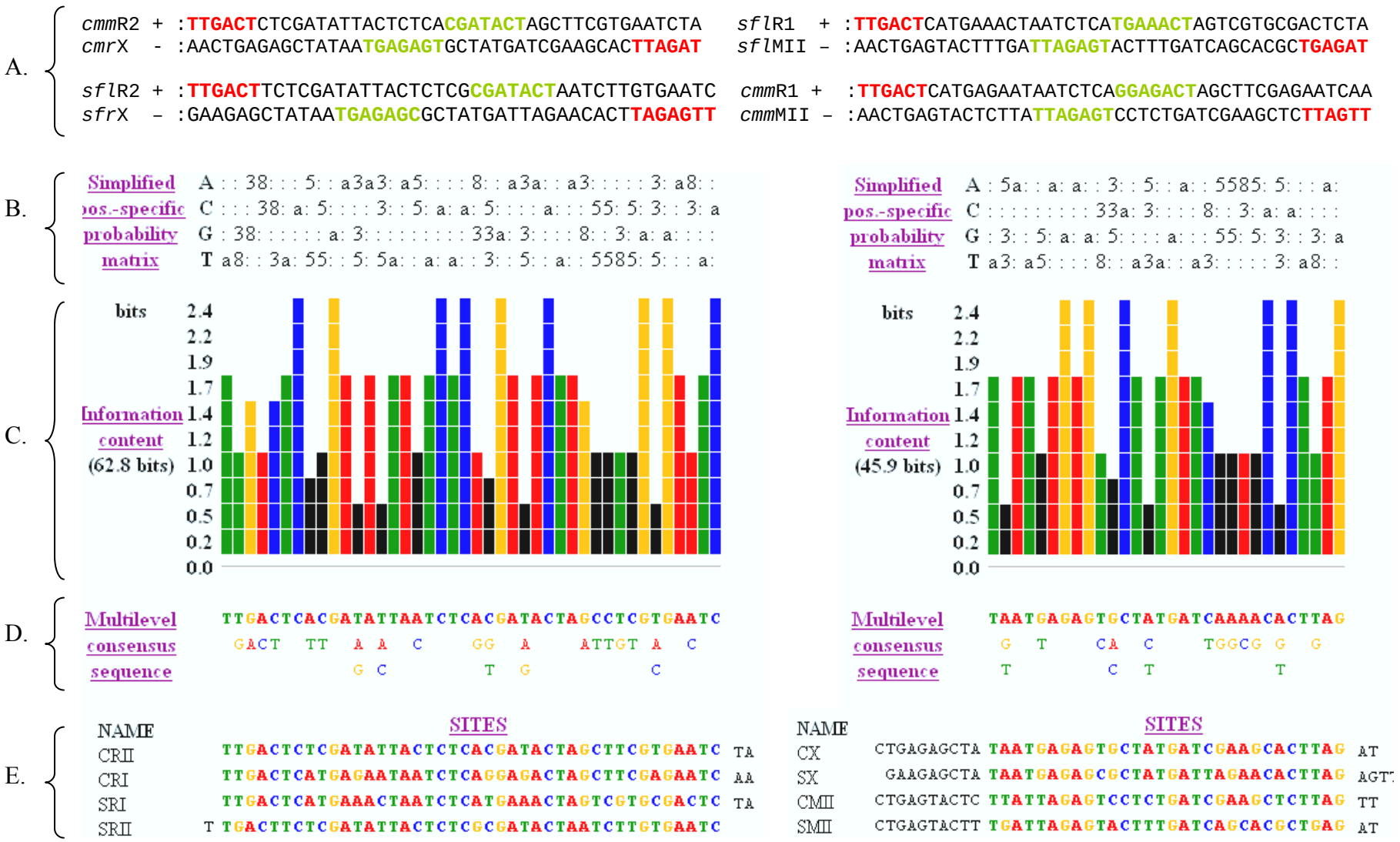


Fig- 7b.7: Pattern generated using MEME software, for bidirectional promoter complexes associated with both regulatory genes of chromomycin synthase clusters of the two producers. A promoter boxes with -35 and -10 box. B Simplified probability matrix. C Diagram showing degree of conservation at each motif. D. Multilevel concensus sequence showing most conserved letter at each motif position E. Aligned sequences with their respective names.

Disruption of *cmm*RII (*sfl*RII homolog) in *S. griseus* not only overproduced chromomycin precociously but was also reported to impart increased resistance against mithramycin, even without induction (Menendez et al, 2007). SflRII another regulatory protein in the cluster was also equipped with promoter complex, with characters very similar to that of *actRp*. It possessed stretch of direct repeats and another promoter region on the opposite strand for SfrX. The promoter of SflRII showed high level conservation to its counterpart (*cmm*RII promoter) from *S. griseus.* Thorough pattern analysis of the promoter complexes of two regulatory proteins from both chromomycin clusters, using MEME software (http://meme.sdsc.edu/meme/meme.html) provided a better understanding of them. (Fig- 7b.7). It also revealed presence of 17 nucleotide spacer in SflRII promoter instead of usual 16 nucleotide spacer present in CmmRII.

These transcriptional activators contain a winged helix-turn-helix motif towards their N-termini which they appear to use in recognizing heptameric repeats within the promoter regions of genes that they regulate (Wietzorrek and Bibb, 1997; Lombo et al, 1999; Sheldon et al, 2002). But ironically in this case, the regulatory proteins themselves possess such promoter sequences with direct repeats in their promoters. Thus, it appears that the two regulatory proteins (SflRI and SflRII) besides directly regulating chromomycin biosynthesis are also influencing their own or each other's expression.

There can be variation in expression levels of the proteins when compared to those in *S. griseus* owing to the small changes in promoter regions because alteration in the spacer region significantly affects promoter activity (Strohl, 1992). Thus, looking at the complexity involved in promoter regions and the nature of overall regulation demands a dedicated study of the same.

Unfortunately all efforts of a successful recombinant of *S. flavisclerotic*us failed. This could be attributed to a very poor transformation frequency encountered with the strain.

With reference to the low number of exconjugants achieved in site specific recombination event of *actRp*-Pij8600 earlier, chances of achieving exconjugant via homologous recombination is scarce.

CONCLUSION

Heterolgous expression of putative genes namely *sfr*A, *sfr*B and *sfr*X proved their role of imparting self-resistance to the cell against chromomycin. SfrAB pump showed a much stronger resistance and appeared to be the playing major role in determining resistance by causing extrusion of the molecule. SfrX alone could impart low level resistance and plays rather a supportive role by taking care of damage, caused by the molecules that escape SfrAB action.

The complex promoter region of *actRp* with a 12 base direct repeat and two 10 base inverted repeats makes it a potential site for SARP binding site. This is evidenced by its ability to express in *S. lividans* and not in *S. flavisclerotic*us, when cloned upstream of EGFP. Lack of pathway specific inhibitory protein in the heterologous host could have allowed functioning of the promoter but not in its native host.

Disruption as well as overexpression of SflRI could have thrown much light on this regulation system, but for the poor transformation efficiency of the strain which did not support the homologous recombination. Though recombinants for SflRI could not be achieved, the single step strategy for disruption and complementation appears to be promising, particularly for studying essential nature of genes and achieving their disruption.

A great deal of information is generated from the present study of genes, involved in resistance as well as regulation of chromomycin biosynthesis. Though these genes are not directly involved in biosynthetic process but occupy key functional positions. There appears to be a crosstalk between these two groups of genes wherein the regulatory genes have some control over expression of resistance genes and on other hand protein involved in resistance is also speculated to have a regulatory role. From the results, there emerges a highly complex system of regulation and resistance whose role might not be restricted only to chromomycin production but might have a wider direct or indirect influence over other processes in the cell. An in-depth study exclusively for the regulatory system of

chromomycin biosynthesis would probably unfold new and far reaching role of these genes of antibiotic cluster with wider implications than expected.

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