

## Chapter -5

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### *Comparative Phylogenetic Studies of*

*S. flaviscleroticus*

&

*S. griseus* sub *griseus*

## INTRODUCTION

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The discovery of antibiotics produced by Streptomycetes in the 1940s led to extensive screening for novel bioactive compounds and subsequently the need for patenting led to an over-classification of the genus. Producers of novel natural products were described as new species and patented. Species described within the genus *Streptomyces* increased from approximately 40 to over 3000. Many of these strains were considered to be synonymous. Standard identification criteria and type strains were needed to prevent overspeciation. In 1964, the International *Streptomyces* Project (ISP) was initiated to introduce standard criteria for the determination of species so as to reduce the number of poorly described synonymous species. Shirling & Gottlieb (1968, 1969, 1972) described the standard criteria that involved the use of spore chain morphology, spore surface ornamentation, color of spores, substrate mycelium and soluble pigments, production of melanin pigment and the utilization of a range of carbon sources. More than 450 *Streptomyces* species were redescribed and type strains were selected and deposited in internationally recognized culture collections. The ISP did not provide an identification scheme, but only standard methods, by which one could be achieved. Williams *et al.* (1983) used a numerical taxonomic approach based on phenetic characters which resulted in a reduction of the numbers of described *Streptomyces* species, the 1989 edition of *Bergey's Manual* describes 142 species (Williams *et al.*, 1989), in contrast to 463 species described in the 1974 edition (Pridham & Tresner, 1974). This did not fully resolve the problem of overspeciation, numerous species and subspecies were described and many natural isolates did not match the reference strains used to construct the identification matrices (Goodfellow & Dickenson, 1985). Additional chemotaxonomic and molecular methods are now used together with the numerical taxonomic methods to improve our understanding of species relatedness within the *Streptomyces* genus. These include cell wall composition (Lechevalier & Lechevalier, 1970), phage typing (Wellington & Williams, 1981), DNA-DNA hybridization (Labeda, 1992), ELISA (Kirby & Rybicki, 1986), rapid biochemical assay for utilization of 4-methyl-umbelliferone-linked substrates (Goodfellow *et al.*, 1987),

comparison of ribosomal protein patterns (Ochi, 1989), low-frequency restriction fragment analysis (Beyazova & Lechevalier, 1993), and comparisons of 16S rRNA and 23S rRNA sequences (Stackebrandt *et al.*, 1991).

The present study has been designed to answer the questions arising from the two different organisms producing the same antibiotic. The molecule is very complex and over 20 genes are involved in its synthesis (Menendez et al, 2004). The primary aim of the present study is to establish the phylogenetic proximity of the two chromomycin producers and to find justification for the presence of same polyketide in the two different species. This could be explained by two different hypotheses:

- 1) Both species are descendents of a common chromomycin producing ancestor and during course of evolution of both the species, the cluster remained conserved.
- 2) The chromomycin gene cluster has been acquired by horizontal transfer by a) from a common donor or b) from either organism to the other.

## **RESULTS & DISCUSSION**

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### **5.1 Relative response to different growth medium:**

It is well known that an organism may respond variedly when cultured on different media. This depends on the requirements of the organism and the constituents of the media (Zhuang, 2006). We observed that both the species showed difference in morphology and metabolite production, when grown on various media, but the important point was the marked difference in the growth pattern of the two species when cultured on the same medium.

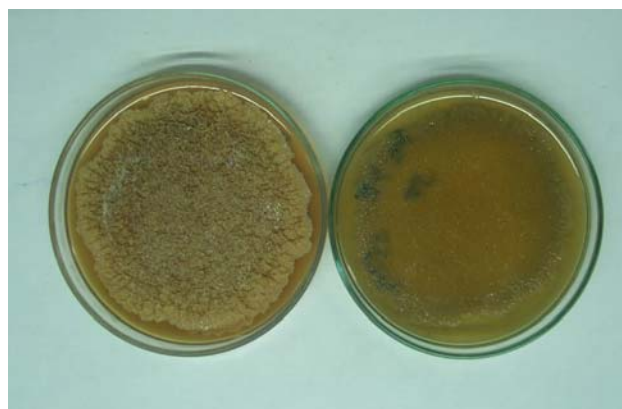
When grown on Soyabean meal Mannitol Agar medium, there was moderate mycelial growth of both strains. There was no sporulation noted after two days of incubation, but the medium turned light green in colour, which could be attributed to the metabolite production including chromomycin which is hallmark of these strains and known to be produced on this medium. After four days of incubation, there was profuse growth and sporulation, covering the mycelium and forming a matt over the top of its surface turning plate white in colour.

On Bennett's Medium, mycelia revealed expected profuse growth pattern in both the species but *S. flaviscleroticus* failed to sporulate even after four days. This could be either due to richness of the medium or due to the uncertain behavior for sporulation observed in this species. The metabolite production appeared to be different in both the species as marked by the difference in the colour of the medium. Also, the colouration was pronounced at the center in *S. flaviscleroticus* whereas it was towards the periphery in *S. griseus* as seen in figure – 2.1.

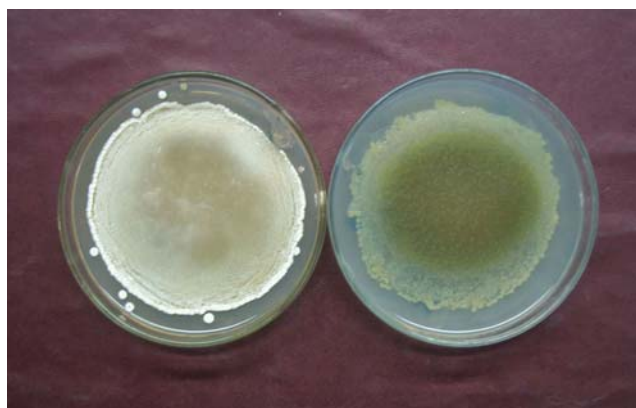
Tryptone Soya Agar supported the growth of mycelium very well for both the species and there was a star shaped growth pattern of *S. griseus* mycelium, which could be attributed to the overgrowing mycelium. The mycelium remained white revealing no



**R<sub>2</sub> Medium**



**R2YE**



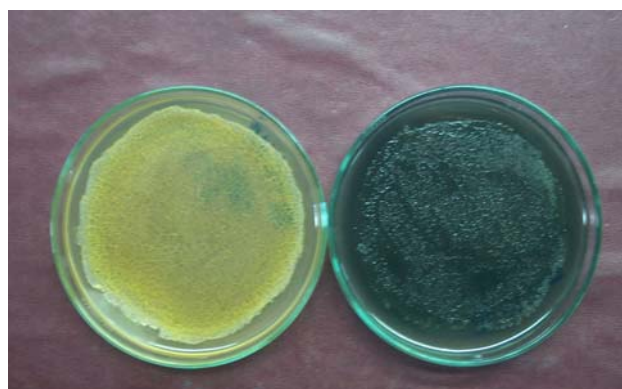
**Bennett's Medium**



**SM Medium**



**TSA Medium**



**YEME Medium**

Fig- 5.1: Growth of *S. griseus* (left) and *S. flaviscleroticus* (right) on different media.

Table – 5.1: Response of *S. flaviscleroticus* and *S. griseus* to various growth media at two time intervals.

Medium	Parameter	After 48 hours		After 96 hours	
		<i>S. flaviscleroticus</i>	<i>S. griseus</i>	<i>S. flaviscleroticus</i>	<i>S. griseus</i>
R <sub>2</sub>	Growth	+	+++	+	+++
	Mycelium	+	+	+	+
	Colour	White	White	White	White
	Spore	+	+	+	+
R <sub>2</sub> YE	Growth	+++	+++	+++	+++
	Mycelium	Star like	Star like	Star like	Star like
	Colour	Yellowish green	Dull orange	Yellowish green	Dull orange
	Spore	-	+	-	+
YEME	Growth	+++	+++	+++	+++
	Mycelium	Shining black	Web like	Shining black	Web like
	Colour	Black	Golden yellow	Black	Golden yellow
	Spore	-	-	-	-

Contd...

Table – 5.1: Response of *S. flaviscleroticus* and *S. griseus* to various growth media at two time intervals.

Medium	Parameter	After 48 hours		After 96 hours	
		<i>S. flaviscleroticus</i>	<i>S. griseus</i>	<i>S. flaviscleroticus</i>	<i>S. griseus</i>
Bennet's Agar	Growth	+++	+++	+++	+++
	Mycelium	+	+	+	+
	Colour	Light green	Yellow/brown	Light green	Yellow/brown
	Spore	-	+	-	++
Soyabean Meal Mannitol Agar	Growth	++	+++	+++	+++
	Mycelium	+	+	++	++
	Colour	Light green	White	Light green	White
	Spore	+	+	+	+
Tryptone Soyabean Agar	Growth	+++	+++	+++	+++
	Mycelium	+	Star like	+	Star like
	Colour	Cream	White	Cream	white
	Spore	-	+	-	-

intracellular accumulation of any detectable metabolites in the cell. Similar pattern of star shaped mycelial growth was also observed for both the species when cultured on R<sub>2</sub>YE medium. There was a difference in pigmentation of both species, while *S. flaviscleroticus* was yellowish green in colour, *S. griseus* developed a dull crimson colour. R<sub>2</sub> which is considered to be the minimal medium for *Streptomyces* culturing, also supported a slow growth and sporulation after four days of incubation. Moreover, there was no conspicuous pigmentation developed either in mycelium or growth on solid agar. The most striking difference was observed on the cultures grown in YEME medium. *S. flaviscleroticus* culture turned dark and was appearing almost black in colour due to excessive production of metabolites while *S. griseus* medium developed a prominent golden yellow colour.

Thus, this study clearly suggests difference between the nutrient requirements of the two species and implies that these two chromomycin producers differ from each other in their response to different media.

## 5.2 Comparative analysis of secondary metabolite signatures

Information derived from chemical analyses of cell extracts has been frequently used for classification and identification to assign suprageneric affinities of the actinomycetes taxa. (Hamid et al, 1993). Owing to the striking difference in the metabolites leached in medium by the two species in study, an attempt was made to scan the spectral signatures of the methanolic crude extracts. A complete spectral scan from 200nm - 700nm involving both, ultraviolet as well as visible light was carried out. The mild yellow and orange coloured extracts did not show any peaks past 550 nm and so the region beyond it was not included in the graphs. Also there was heavy absorbance in the ultraviolet region as compared to the visible region in both the extracts (fig – 5.2). Absorbance of *S. flaviscleroticus* extract was found to be relatively higher in most cases, to that of *S. griseus*. For YEME extracts, owing to abundant metabolite production, the concentrations of extracts were appropriately diluted before use, the absorption in the



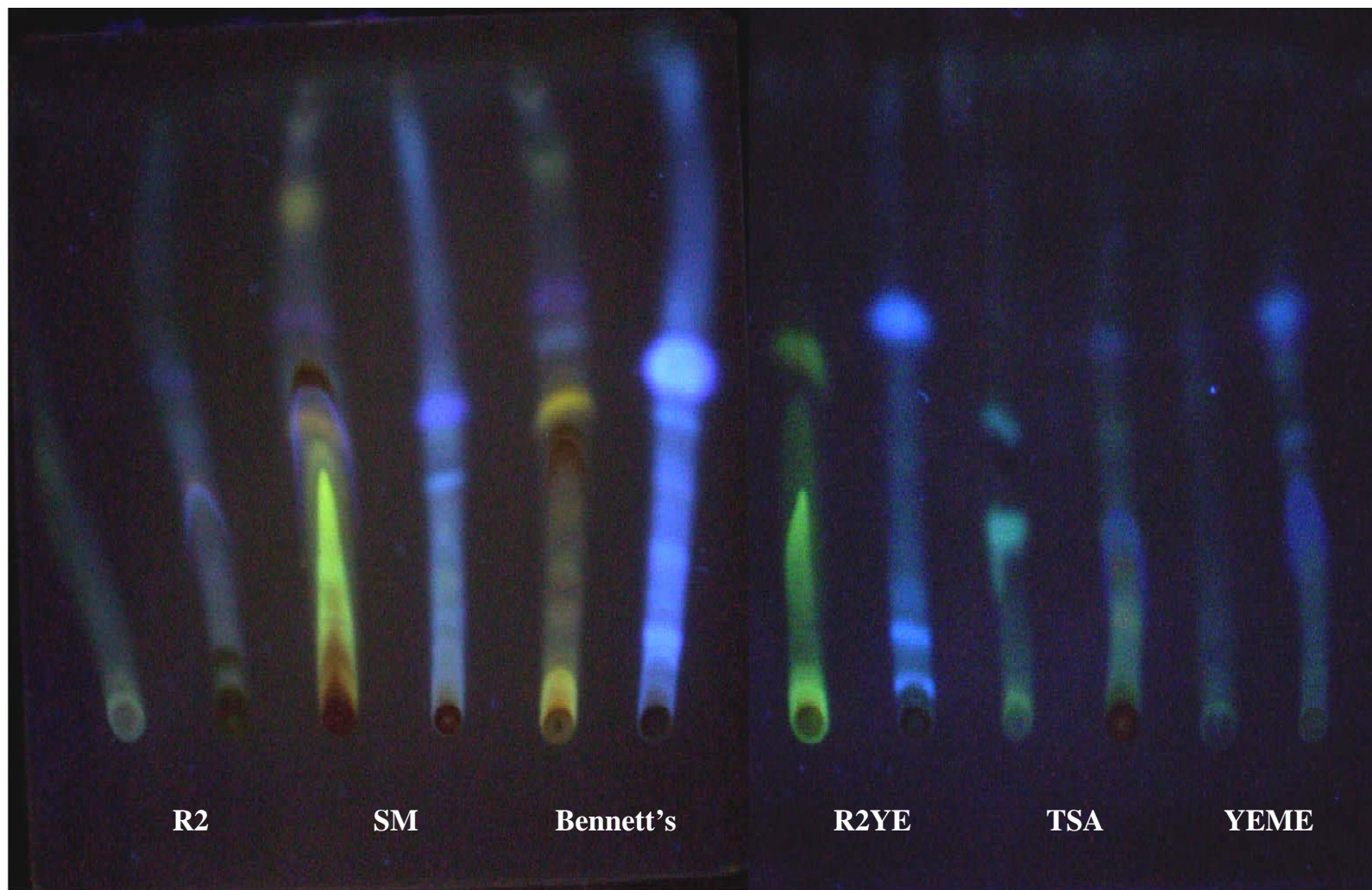


Fig –5.2: Comparative TLC of metabolite extracts of *S. flaviscleroticus* (left) and *S. griseus* (right) from various media, under uv light (365nm)

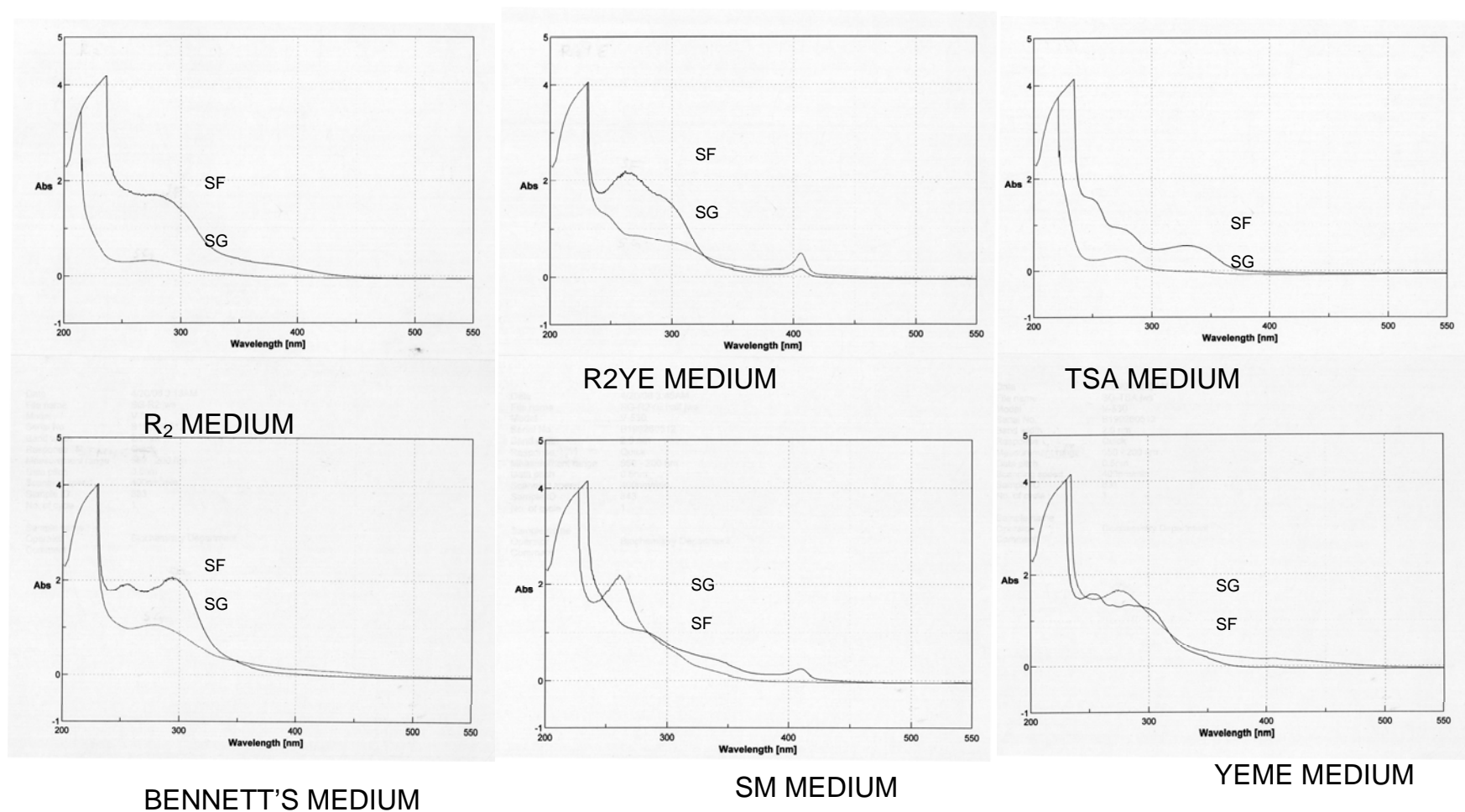


Fig – 5.3: Relative spectrophotometric signatures of *S. flaviscleroticus* (SF) and *S. griseus* (SG) extracts from different media

ultraviolet range crossed the maximum detectable range of the spectrophotometer (Helios Gamma).

Importantly, for most of the media, growth of *S. griseus* was visibly better than *S. flaviscleroticus*, but the amount of metabolites produced in the same media was more in *S. flaviscleroticus* and thus can be a better source of metabolites. The separation pattern of the metabolites from microorganism on thin layer chromatography plates, as well as their chemical reactivity towards some staining reagents under defined reaction conditions enables the visualization of an almost complete picture of the secondary metabolites, also known as their metabolic fingerprint. (Burkhardt et al, 1996). The results of thin layer chromatography obtained from methanolic crude extract of both the taxa cultured on different media were quite convincing in relation to the results obtained in spectral studies.

After chromatographic separation on TLC plates, very few coloured spots could be observed. This is in correlation with the spectral data, most of the peaks were obtained in the ultraviolet range. So instead of using the popular technique of staining TLC plate with different reagents for developing spots, TLC plates were directly observed under uv light and several metabolites fluoresced brightly as seen in fig– 5.3. Further, the fluorescence was also of characteristic colours wherein, *S. flaviscleroticus* spots were dominated with bright blue fluorescence away from the base whereas there was dominance of green fluorescence towards the base in *S. griseus* extracts.

### 5.3 Restriction Endonuclease Analysis (REA)

Restriction endonuclease analysis of genomic DNA is a popular technique and has been used to classify various bacterial strains within the genus *Campylobacter* (De Lisle et al., 1987), *Clostridium* (Devli et al., 1987), *Escherichia* (Marshall et al., 1985), *Legionella* (Vankatel 1988), *Mycobacterium* (Collins and De Lisle, 1985), *Neisseria* (Bjorvatan et al., 1984), *Staphylococcus* (Renaud et al., 1988) and *Streptococcus* (Skjold et al., 1987). For *Streptomyces*, the technique has been adopted to distinguish between

*Streptomyces coelicolor*, *S. noursei*, *S. parvulus*, *S. peucetius* and *S. scabies* (Crameri *et al.*, 1983) and even for distinguishing mutants of *S. glaucescens* (Hintermann *et al.*, 1981).

When digested with *Bgl*II, *Bam*HI, *Hind*III and *Pst*I enzymes the taxa under study showed considerable mismatch in RE digestion pattern of genomic DNA. If the two taxa were close relatives, they would have shown relatively similar banding pattern, if not identical. But the incongruity in the pattern reflects that the two systems are much different from one another or that the taxa have acquired several mutations to variably respond to the restriction enzyme digestion.

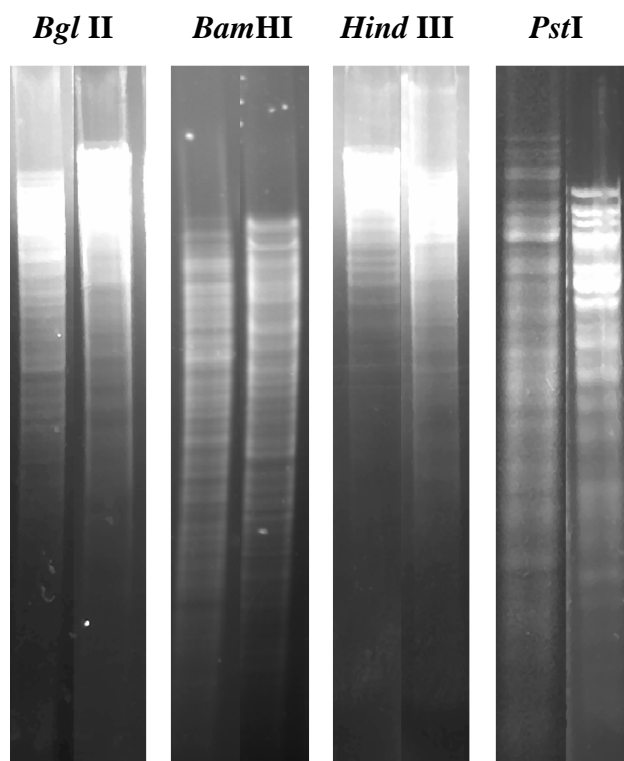


Fig – 5.4 : Restriction Enzyme Digestion Pattern of Genomic DNA from *S. flaviscleroticus* (left) and *S. griseus* (right) using different enzymes.

#### 5.4 Response to Salt and pH stress

There was a notable difference observed in the ability of *S. flaviscleroticus* and *S. griseus* to withstand the osmotic stress generated due to high concentrations of NaCl in the medium. From the data (Table-5.2) it is evident that *S. griseus* has a stronger resistance, of the two species under study, and could show growth even at salt concentrations of as high as 7% as against *S. flaviscleroticus* that struggled to withstand even 5% of salt stress. Since the growth of *S. griseus* diminishes with increasing salt concentration, it can be categorized as relatively more halotolerant but not halophilic i.e. it recognizes high salt concentration in media as an adverse condition and tolerates it but it does not prefer to grow on it.

It was observed that the plates inoculated with *S. flaviscleroticus* started turning dark and the effect gradually pronounced with time and after four days there was a significant pigmentation developed in medium, an effect which was not found in *S. griseus*. This could have been a result of a stress response sensed by *S. flaviscleroticus*, which could have upregulated production of certain metabolites, leached in the medium. The differential expression of genes encoding for certain metabolites as a response to salt stress has been reported in *S. coelicolor* A3 (2) (Beatrica and Kormanec, 2004)

Increase in free intracellular amino acids and other solute concentration, proline in particular increases efficiency to grow in high salt conditions (Killham and Firestone, 1984). *S. griseus* could have adopted some similar mechanism for its salt tolerance phenotype. Sporulation normally occurs when there is an adversity felt by the organism but in this case there was no sporulation observed in either organism at any salt concentration.

Optimum pH for growth of *Streptomyces* sp. is 7.2 as observed in most media preparation (refer materials and methods). Any deviation from this can significantly affect growth of the organism. For both the strains under study, minimum pH that could

Table – 5.2: Response of *S. flaviscleroticus* and *S. griseus* to increasing salt stress measured at two intervals

Salt concentration	Parameter	After 48 hours		After 96 hours	
		<i>S. flaviscleroticus</i>	<i>S. griseus</i>	<i>S. flaviscleroticus</i>	<i>S. griseus</i>
5 %	Growth	-	++	++	+++
	Sporulation	-	-	-	+
6 %	Growth	-	++	+	++
	Sporulation	-	-	-	-
7 %	Growth	-	++	+	++
	Sporulation	-	-	-	-
8 %	Growth	-	-	-	+
	Sporulation	-	-	-	-

Table – 5. 3: Response of *S. flaviscleroticus* and *S. griseus* to varying pH measured at two intervals

pH	Parameter	After 48 hours		After 96 hours	
		<i>S. flaviscleroticus</i>	<i>S. griseus</i>	<i>S. flaviscleroticus</i>	<i>S. griseus</i>
4	Growth	-	-	-	-
	Sporulation	-	-	-	-
5	Growth	- / +	-	+	++
	Sporulation	-	-	-	-
6	Growth	++	++/+++	++/+++	+++
	Sporulation	-	+	+	++
7	Growth	++	+++	+++	+++
	Sporulation	-	++	-	++

allow the growth was found to be pH 5 (Table – 5.3). Neither strains could tolerate pH lower than that as marked by absence of growth in pH 4. Thus, there was no significant difference in pH tolerance levels of *S. flaviscleroticus* and *S. griseus* but at the same time, there were differences observed within the tolerance limits. *S. griseus* showed profuse growth after four days at pH 6 whereas *S. flaviscleroticus* could grow moderately. Also, *S. griseus* could sporulate at every pH where it could grow, but *S. flaviscleroticus* sporulation was inhibited below pH 6. Thus *S. griseus* was relatively more robust than its counterpart with respect to pH parameter on the scale.

### 5.5 Molecular Phylogeny Studies

The primary, secondary and tertiary structures of rRNA molecules have remained conserved during evolution (Gutell, 1992). Analysis by classical methods of comparative oligonucleotide cataloguing and consideration of full or partially complete sequences of 16S rRNA have revealed that the primary structure of rRNAs consists of highly conserved regions interspersed by regions of moderate to low homology within related species (Gopo et al, 1988). Despite the highly conserved nature of rRNAs, they vary in size and in the organization of the spacer as well as variable regions within the rRNA. The small size of 5S rDNA and extensive secondary and tertiary structures present in the 23S rDNA render these molecules unsuitable for sequencing and further analysis (Anderson and Wellington, 2001). However, the moderate size of the 16S rDNA, sequence conservation during evolution and confirmed importance in classification has proven that 16S rDNA sequences can be used effectively for phylogenetic identification of the microorganisms at various levels of hierarchy (Anderson and Wellington, 2001). For this reason, the most acceptable method of 16S rDNA comparison was carried out to study the relatedness of the two chromomycin producing species of *Streptomyces*, namely *S. griseus* and *S. flaviscleroticus*.

Annealing temperature for the primers used in the study was kept at 60 °C in thermocycler (mastercycler, eppendorf) with a gradient of 5 °C. Thus, annealing temperatures between 55 °C and 65 °C were tested in single run and the amplicon was

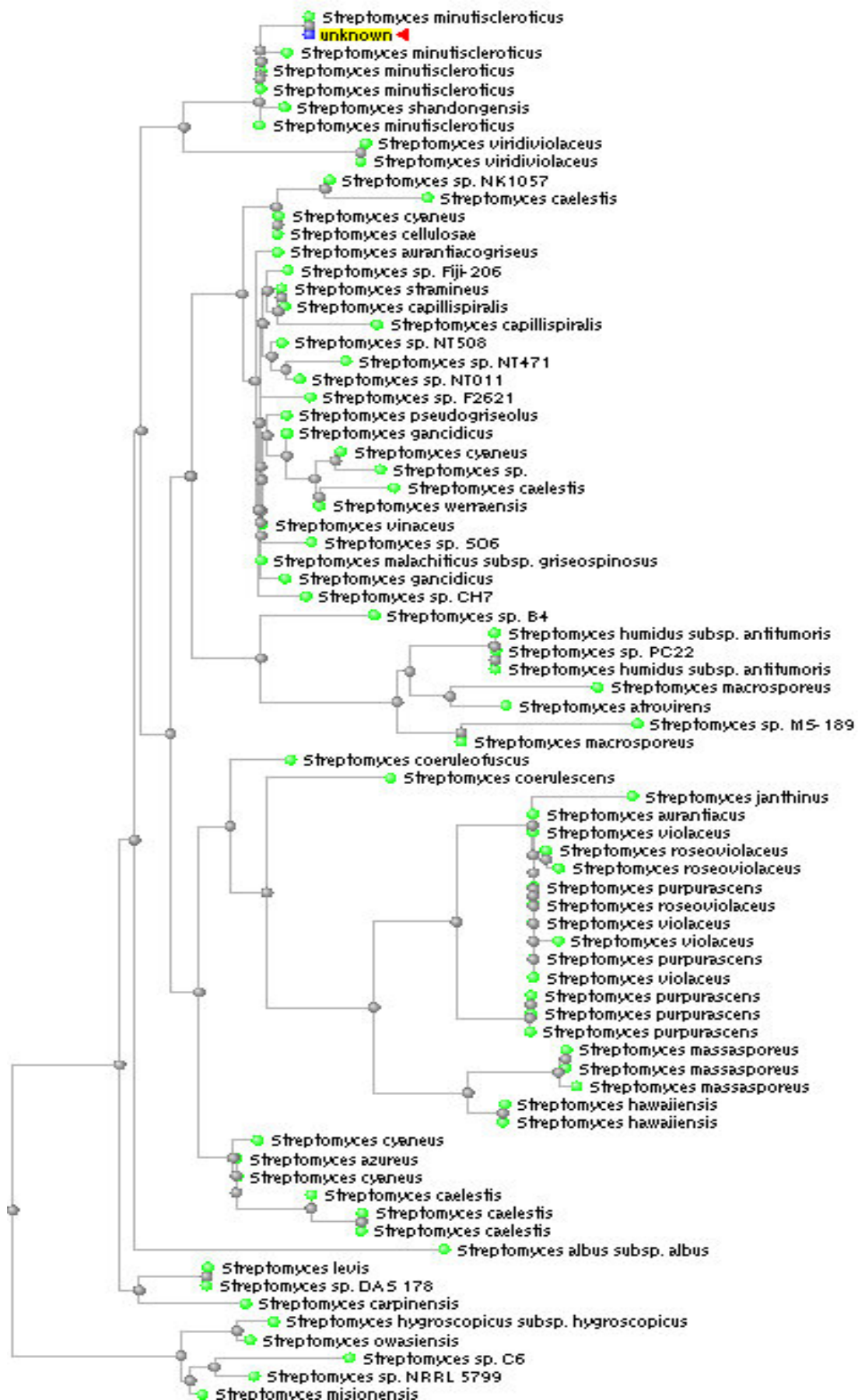


Fig 5.5 : 16S rDNA phylogenetic tree of *S. flaviscleroticus*



best obtained at 56 °C. For cloning the amplicon, pBSK was selected due to the presence of T3 and T7 regions, flanking the MCS that are used as standard primers for DNA sequencing.

BLAST search for *S. flaviscleroticus* 16S rDNA sequence was carried out using nucleotide-nucleotide BLAST. It revealed a close homology with various species of *Streptomyces* like *S. viridiviolaceus*, *S. levis*, *S. cellulosa*, *S. aurantiacogriseus*, *S. cyaneus*, *S. griseospinosus*, *S. werraensis*, *S. violaceus*, *S. purpurascens* and several others. The data was then subjected for generation of a phylogenetic tree view on the basis of evolutionary pattern and species similarity as seen in the figure – 5.5. The divisions and distances show the relatedness of *S. flaviscleroticus* with other *Streptomyces* sp. The closest species to the one under study was found to be *S. minutiscleroticus*. Surprisingly, *S. griseus* was not among the 40 species present in the tree of *S. flaviscleroticus*. *S. griseus* did not light up in the tree even at the highest mismatch freedom of 7.5 units. The tree of *S. griseus* suggested species like *S. lavendulae*, *S. flavogriseus*, *S. argenteolus*, *S. setonii*, *S. albus*, *S. anulatus* to be close relatives. The tree included around 67 species that had high homology in 16S rDNA sequences with *S. griseus* which did not include *S. flaviscleroticus*.

The phylogenetic tree revealed the degree of unrelatedness between the two species and so to further quantify this incongruity nucleotide-to-nucleotide homology was checked using BLAST2SEQUENCES software, available at NCBI portal. The results gave a final picture of the unrelatedness of the two species. The data obtained is as follows:

Expect	=	0.0
Identities	=	1416/1488
Gaps	=	32/1488

The expect value of 0.0, which means that there is zero chance event (probability) for the two sequences to be coding for different genes. The homologous source of the sequence is already known and so for this particular study it should be ignored. It could

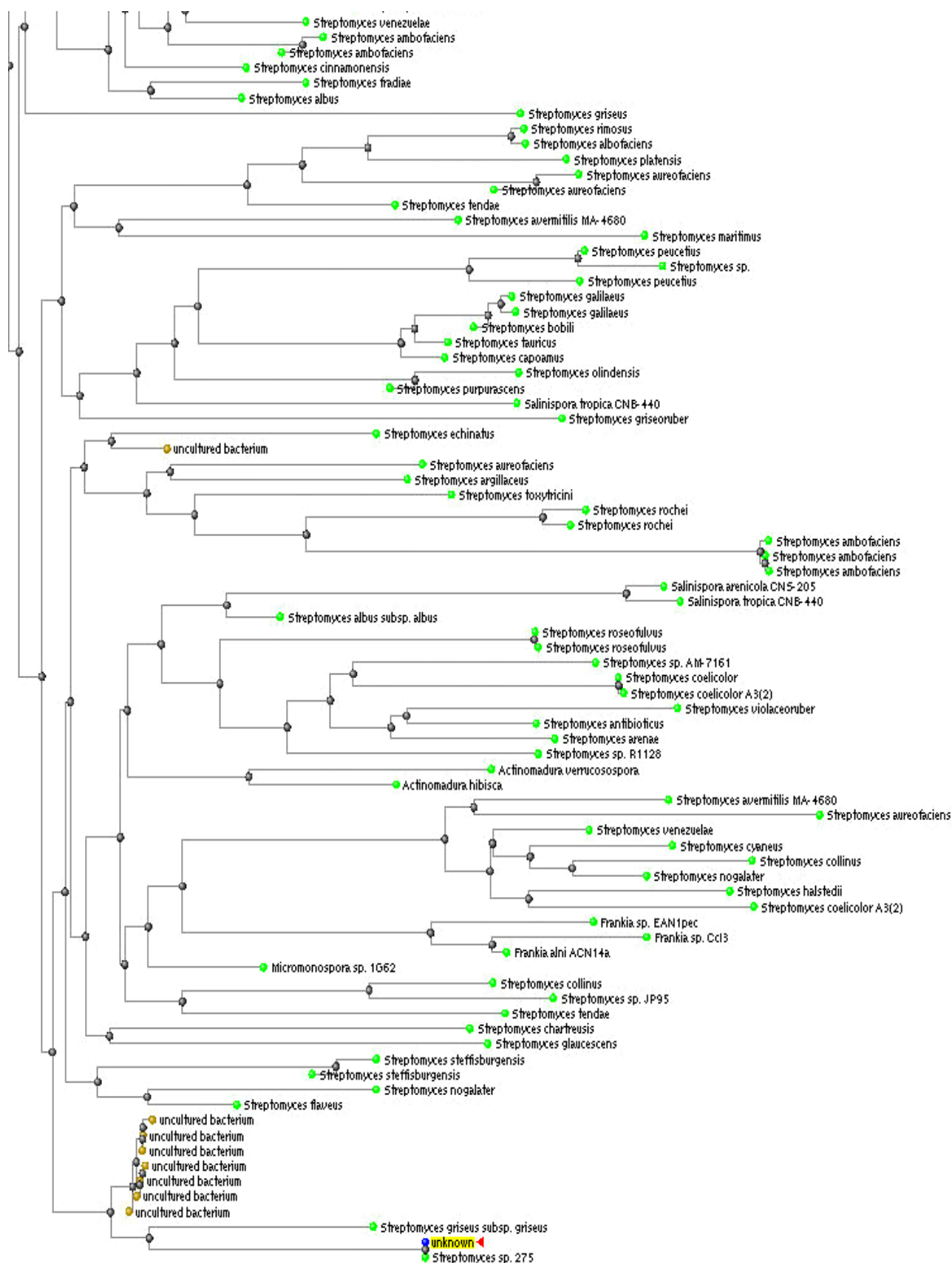


Fig – 5.6 : Phylogenetic tree of ketosynthase gene of *S. flaviscleroticus*

have been of greater impact if an unknown sequence under study was being compared to a known one available in database or otherwise.

Interestingly, there are 32 gaps between the two sequences, which accounts to over two percent region of 1.488kb DNA sequence. Moreover, these gaps could include one or more nucleotide(s) and thus the actual dissimilarity can be determined by 'identities' between two DNA sequences. Total identities are of 1416 bases out of 1488 bases. Thus there is a direct mismatch of 72 bases, accounting to around 5% mismatch between the two sequences. The mismatch can be in the form of an additional or a missing nucleotide, or it can be due to any other nucleotide present at that position. The stretching of the sequence that has originally 1472 bases to 1488 in the identities figure reflects net presence of 16 additional bases in the two strands. This search justifies the data from phylogenetic tree that suggested *S. flaviscleroticus* and *S. griseus* to be distant relatives and provides confirmative results on the same.

Metsa-Ketela et al. (2002) has developed a method that could be used for classification of strains on the basis of their characteristic genetic abilities to produce various compounds belonging to the aromatic polyketide group. This method is based on studying deviation in conserved ketosynthase gene, which is an essential component of 'minimal PKS', a cassette of genes for the biosynthesis of aromatic polyketides. On phylogenetic analysis of ketosynthase sequence, it was revealed that the two chromomycin producers formed a separate clade and there was no other known close relative of these two producers in it (fig 5.6). Other members included in the clade consisted of all uncultured bacterium. *S. argillaceous* producer of another aureolic acid antibiotic - mithramycin, was placed far away on a separate branch in the phylogenetic tree. Atleast this case turns down the proposal made earlier (Metsa-Ketala et al, 2002) that organisms producing molecules of same group (aureolic acid, in this case) are present on the same branch in the clade. Further, it also suggested that any disparity in this generalized trend could result due to acquisition of DNA laterally, which strengthens our view.

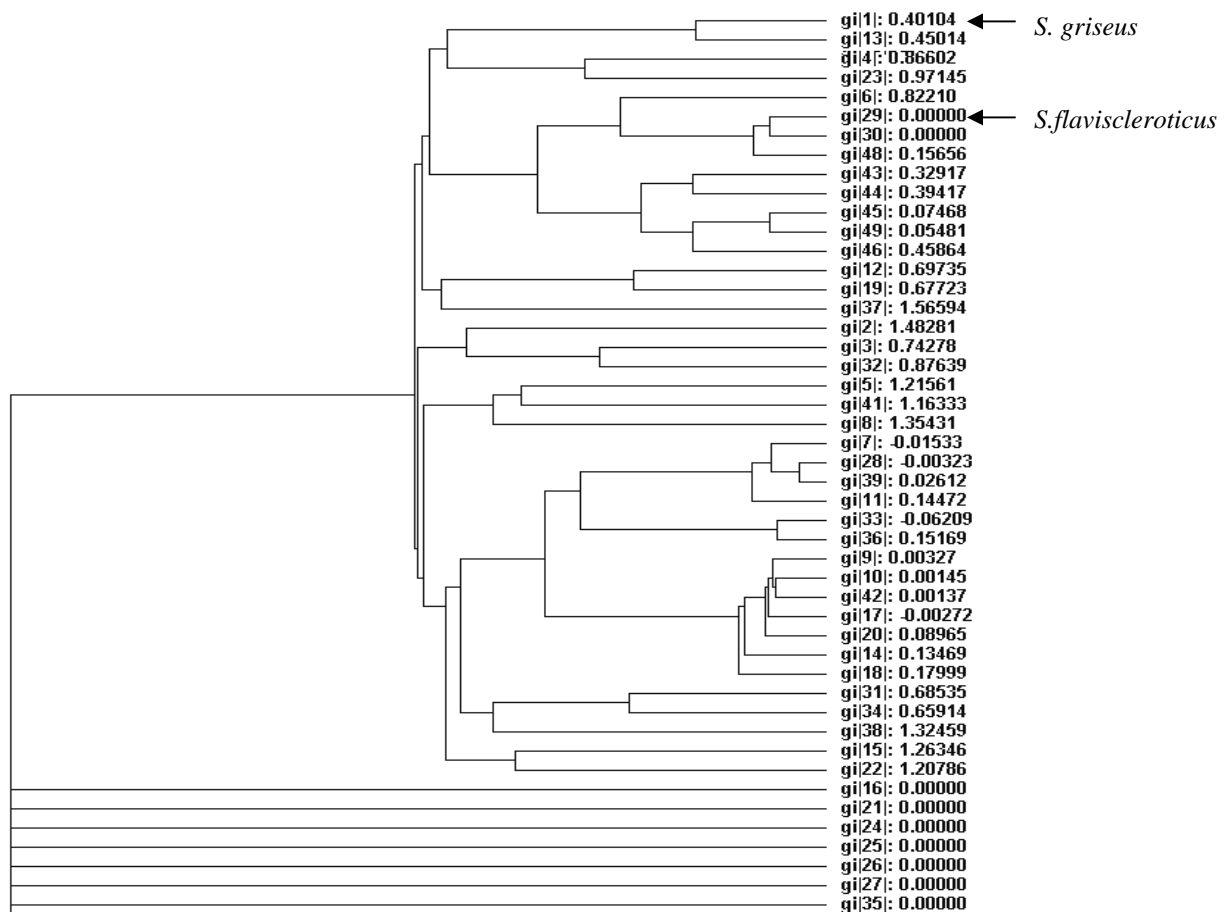


Fig-5.7: CLUSTLW phylogram of selected 16S rDNA sequences

The anomaly in the results of the two methods used above could be due to huge difference in the richness of database of the two genes. While 16S rDNA database is exhaustive, ketosynthase database is restricted due to the difference in the prevalence of the two genes. To check if the difference in results was attributed to horizontal transfer of the cluster or is a result of technical limitation, a 16S rDNA phylogenetic tree of only those organisms that were present in ketosynthase phylogenetic tree, was prepared using CLUSTLW software.

As seen in the data (fig 5.7), this rules out the earlier doubt and confirms the results that the two species are not close relatives as could have been hypothesized due to presence of same polyketide producing cluster in both the species.

## CONCLUSION

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The morphological, cultural, biochemical studies advocate that the two chromomycin producing species namely, *Streptomyces flaviscleroticus* and *Streptomyces griseus* are not same and significantly differ from each other owing to the differences observed in several aspects under study. Results of *in silico* studies using 16S-rDNA region clearly showed the two species to be distant from each other. The only common factor observed between the two species during entire study was the type II polyketide – Chromomycin and close proximity of conserved Ketosynthase genes of the two species in the cladogram.

This result can only be explained by horizontal gene transfer theory, wherein the PKS cluster of chromomycin must have been acquired by either species from the other, or by both from common ancestor via a mobile carrier like plasmid, transposon or phage which might have later stabilized itself in the genome.

The results are substantially supported by the later work (chapter-3) involving sequencing of PKS cluster DNA. It was found that not only the conserved ketosynthase, but the entire cluster has very high homology with that of *S. griseus* sub *griseus*. Further, the organization, positioning and orientation of genes in the cluster were also found to be identical. Also presence of transposon remnants at one end of the cluster and a transposon within the cluster (discussed in chapter-3) revealed from sequencing strengthens the theory.

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